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Molecular Cloning of Plasmolipin

CHARACTERIZATION OF A NOVEL PROTEOLIPID RESTRICTED TO BRAIN AND KIDNEY*

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From the ‡Department of Anatomy and Neurobiology, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129, the §Division of Neurobiology, N. S. Kline Institute, Orangeburg, New York 10962, and the ¶Department of Psychiatry, New York University, New York, New York 10016

Plasmolipin is an 18-kDa proteolipid protein found in kidney and brain, where it is restricted to the apical surface of tubular epithelial cells and to mammalian myelinated tracts, respectively (Sapirstein, V. S., Nolan, C. E., Stadler, I. I., and Fischer, I. (1992) *J. Neurosci. Res.* 31, 96–102; Cochary, E. F., Bizzozero, O. A., Sapirstein, V. S., Nolan, C. E., and Fischer, I. (1990) *J. Neurochem.* 55, 602–610). Addition of plasmolipin to lipid bilayers induces the formation of ion channels, which are voltage-dependent and K^+-selective (Tosteson, M. T., and Sapirstein, V. S. (1981) *J. Membr. Biol* 63, 77–84). The present study describes the isolation and cloning of plasmolipin cDNA that includes the sequence of the complete coding region of the protein, the analysis of plasmolipin mRNA expression and a proposed model for its membrane structure. Northern blot analysis 1) shows that plasmolipin is encoded by a 1.7-kilobase mRNA, 2) confirms that the distribution of plasmolipin is restricted to kidney and brain, and 3) indicates that the expression of plasmolipin mRNA in cultured oligodendrocytes increases with cell maturation consistent with changes in the level of the protein. Restriction enzyme digestion of DNA followed by Southern blot analysis indicates that plasmolipin is encoded by a single gene. Sequence analysis of plasmolipin cDNA shows an open reading frame encoding a 157-amino acid protein of 17.4 kDa. The deduced amino acid sequence confirms the hydrophobic nature and high helical content of the protein and predicts a structure with four transmembrane domains similar to several other small hydrophobic proteins implicated in ion movement. The proposed model for membrane topology shows an enrichment of hydroxyl groups within two of the transmembrane domains and places cysteine residues near the extracellular membrane surface. Examination of protein sequence data bases reveals little overall homology with other proteins including proteolipids; however, three of the four transmembrane segments of plasmolipin show strong similarity with known membrane transport proteins. These results indicate that plasmolipin is an unique proteolipid protein that may participate in ion transport events specific to select membrane domains.

Plasmolipin is a proteolipid protein localized to specific plasma membranes in both kidney and brain (1–6). In the kidney, it is segregated to the apical surface of renal tubular epithelia, where it is concentrated in the proximal segments; plasmolipin is also expressed in renal epithelial cell lines (5). Among the neural cells analyzed *in vitro*, plasmolipin is found only in oligodendrocytes, where its expression increases with cell maturation (6). In brain, it is localized to myelinated nerve tracts and its expression increases markedly with the onset of myelination (5, 6). Membrane isolation, biochemical studies, and immunological studies show that plasmolipin is a myelin protein (3, 4), where it represents 3–5% of the total protein. Plasmolipin is unique among oligodendroglial/myelin proteins by being expressed in comparable amounts in peripheral nervous system myelin (7). Plasmolipin also differs from other major myelin proteins such as the myelin proteolipid protein (PLP),[1] myelin basic protein, and myelin-associated glycoprotein which are segregated to specific myelin domains with plasmolipin present in similar amounts in compact and periaxolemmal myelin (4). Its distribution within myelin appears to include regions active in membrane recycling, since along with the paranodal marker cyclic nucleotide phosphohydrolase, it is enriched in endocytic coated vesicles isolated from myelinated tracts (8–10). These unique characteristics may reflect its functional significance to higher vertebrates with the observation that plasmolipin is restricted to mammals (7).

Proteolipid proteins are a class of hydrophobic membrane proteins distinguished by their solubility in organic solvents (11). These proteins are present in plasma membranes, sarcoplasmic reticulum, endosomal vacuoles, chromaffin granules, and mitochondria (2, 12–16). None of these proteins, including the myelin proteolipid (16), shares significant sequence homology with each other except for the proteolipid of the proton pump of mitochondria and chloroplast, which is the progenitor of that found in vacuoles and chromaffin vesicles with the latter group arising by gene duplication (15). Several proteolipids, including myelin PLP and plasmolipin (2, 16), exhibit a unique structural flexibility, which allows the apoprotein to be soluble in chloroform-methanol or in water. The amphipathic character of proteolipids probably explains their participation in transmembrane ion movement including H^+ (18), Ca^{2+} (19) channels, and possibly in Na^+,K^+-ATPase transport function (20). The biological function of plasmolipin is not known; however, incorporation of the purified protein into lipid bilayers induces voltage-dependent K^+ channel formation, suggesting it may function *in vivo* as an ion channel (1).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U13617.

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[1] The abbreviations used are: PLP, myelin proteolipid protein; H^+-ATPase and Na^+,K^+-ATPase, proton-stimulated and sodium/potassium-stimulated adenosine triphosphatases, respectively; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobase(s).

The present study describes the molecular cloning of plasmolipin, the deduction of its amino acid sequence, and a proposed model for its membrane structure. Southern blot analysis indicates that plasmolipin is encoded by a single gene; Northern blots confirm that plasmolipin is limited to kidney and brain and reveal that its expression in oligodendrocytes correlates with cell differentiation. Analysis of sequence homologies indicates that plasmolipin represents a unique class of proteolipid with little homology with other proteolipids.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against plasmolipin were prepared (21) and affinity-purified as described previously (3). Monoclonal antibodies against bacterial β-galactosidase were purchased from Promega Biotech (Madison, WI).

Gel Electrophoresis and Western Blots—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22) was carried out on 14% acrylamide gel as described previously (21). After electrophoresis proteins were transferred to nitrocellulose membrane using a semi-dry apparatus and were reacted with either anti-plasmolipin or anti-β-galactosidase (1 μg/ml) in 25 mM Tris-buffered saline containing Tris-HCl (Sigma), pH 7.6, Tween 20 (0.05%), and fetal calf serum (0.5%) followed by incubation with a goat anti-rabbit or mouse IgG (1:3,000), conjugated to horseradish peroxide (Bio-Rad). Proteins were visualized by color reaction with diaminobenzidine and hydrogen peroxide (Sigma).

Digoxigenin Detection of Cysteine Residues—A proteolipid fraction was prepared from isolated periaxolemmal myelin (4) as described by Sapirstein and Rounds (2). Proteins were separated on 14% SDS gels under reducing conditions, transferred onto nitrocellulose membranes, and incubated with digoxigenin-3-*O*-succinyl-[2-(*N*-maleimido)]ethylamide to selectively label sulfhydryl groups. The membrane was washed and then incubated with anti-digoxigenin antibodies conjugated to alkaline phosphate. The staining reaction was developed with 4-nitro tetrazolium chloride using 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Screening of cDNA Libraries—The λ gt11 library from rat kidney (1.2 × 10^6 independent clones, average insert size 1.0 kb, Clontech, Palo Alto, CA) was plated on the Y1090 strain of *Escherichia coli* and screened with affinity-purified antibodies against plasmolipin as described previously (23). Positive clones were isolated from the master plates and purified through three additional rounds of screening. Plasmolipin cDNA inserts were excised from recombinant λ gt11 clones by *Eco*RI digestion and cloned into the *Eco*RI site of pGEM-3Z vector (Promega Biotech).

Preparation of the Plasmolipin Fusion Protein—The cDNA clones were expressed in λ gt11 by the modification of the lysogen method as described by Sambrook *et al.* (24). Lysogens were selected and grown on Y1080 to an optical density of 0.5 (595 nm). The fusion protein was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the cultures incubated at 40 °C for 90 min. The cells were harvested, suspended in 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, lysed by freezing and thawing, and prepared for gel electrophoresis.

Preparation of RNA—Total RNA was prepared using the single-step method with guanidium thiocyanate (25) or by LiCl/guanidium thiocyanate (26).

Northern Blots—Radiolabeled DNA probes were synthesized using random primers (27). Total cellular RNA was separated on a 1.2% agarose gels containing 6% formamide, transferred to Zeta Probe nylon membranes (Promega), and hybridized as described previously with the plasmolipin cDNA (23, 26). Integrity of RNA was confirmed by ethidium bromide staining of ribosomal RNA. Each Northern blot was also probed with β-actin or β-tubulin cDNA to ensure the quality of RNA and for use as a normalization reference for the levels of RNA. Quantitative analysis of Northern blots was carried out using the Visage 110 Bio Image System, which detects whole band boundaries through a digitized image (1024 × 1024 pixels) (28). Care was taken to use RNA concentrations within the linear range of detection.

Southern Blots—Genomic DNA was digested by individual restriction enzymes, separated on agarose gels, transferred to nylon membranes, and hybridized with radiolabeled plasmolipin DNA probes as described for Northern blots.

PCR Amplification of DNA—PCR amplification with *Taq* polymerase was used for identification and comparison of clones isolated from the cDNA library (29).

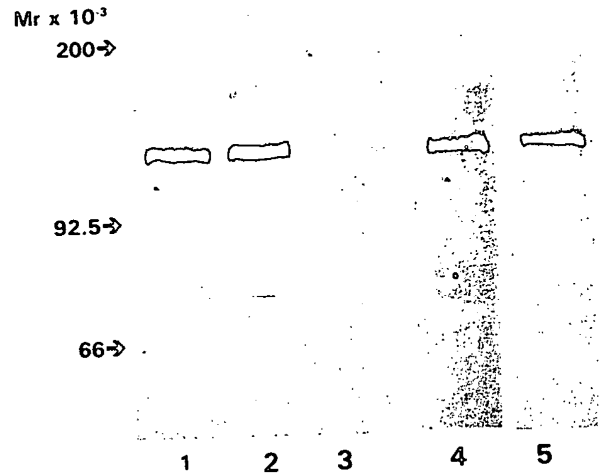

FIG. 1. **Western blot analysis of plasmolipin fusion proteins.** Three clones isolated from a rat kidney cDNA library (pPL1, pPL2, and pPL3) were grown in the presence of IPTG, and the bacterial extracts were analyzed by Western blots with the original plasmolipin antibody that was used for the library screening (*lanes 1–3*, respectively). A new independent affinity-purified plasmolipin antibody was prepared and also recognized the fusion protein (*lane 4*), as did a monoclonal antibody against bacterial β-galactosidase (*lane 5*). The fusion protein size was estimated to be 135 kDa, which is calculated to contain the β-galactosidase with about 15–20 kDa of plasmolipin.

Oligonucleotide Synthesis—The oligonucleotides were synthesized in the Shriver Center molecular biology facility on a Applied Biosystems model 381B DNA synthesizer using standard phosphoramidite chemistry and partial purification (6).

DNA Sequence Analysis—Double-stranded DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.* (30), using the Sequenase kit (U. S. Biochemical Corp.) with modifications essential for double-stranded sequencing. Synthetic oligonucleotides were used as sequencing primers. The DNA sequence of both strands was determined and analyzed with the DNASTAR software package.

Preparation of Primary Rat Glial and Enriched Oligodendroglial Cultures—Primary glial cultures and enriched oligodendroglial cultures were prepared from brains of 3-day-old rats as described previously (31).

RESULTS

Isolation and Characterization of cDNA Clones Encoding Plasmolipin—A λ gt11 rat kidney library was screened using affinity-purified plasmolipin antibodies. Of the 5 × 10^5 cDNA clones that were screened, three plasmolipin-positive clones were obtained, which were purified through three additional rounds of screening. The positive λ gt11 clones (1.2 kb each) were excised with *Eco*RI, and the cDNA inserts (pPL1, pPL2, and pPL3) were subcloned into pGEM-3Z Riboprobe vector. Southern blot analysis using radiolabeled pPL1 as a probe indicated that all the three clones share homology (data not shown), and each of their fusion proteins reacted with affinity-purified anti-plasmolipin (Fig. 1). Subsequent restriction enzyme and PCR and sequence analyses showed the clones to be identical, and we continued to work only with the pPL1 clone.

Characterization of Plasmolipin Fusion Protein—Expression of fusion proteins in selected cDNA clones were induced by IPTG followed by gel electrophoresis and Western blots. The blots were analyzed for the expressed fusion protein of plasmolipin and β-galactosidase with two different plasmolipin affinity-purified antisera (Fig. 1, *lanes 1–4*) and a monoclonal anti-β-galactosidase antibody (Fig. 1, *lane 5*). The fusion protein was recognized both by the original plasmolipin antiserum that was used to screen the library (*lane 1*) and by a second affinity-purified antibody (*lane 4*), supporting the identity of these

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-63      -55          -46          -37          -28          -19          -10              1       9
5'CCG TCG AAA GTG AGC ACC AGG ACC AGC AGC CCC GCG CAG GGC GTG GGA GCA TCT GTG TCT GCG ATG CGA CCG
                                                                                   Met Arg Pro-3

        18          27          36          45          54          63          72          81
GAC CTG GGC TTT GTG CGC TCC GCC CTT GGG GTG CTC GCG CTT CTG CAG CTG GTG CTG GGG CTG CTG GTG TGG
Asp Leu Gly Phe Val Arg Ser Ala Leu Gly Val Leu Ala Leu Leu Gln Leu Val Leu Gly Leu Leu Val Trp-27

        90          99          108         117         126         135         144         153
GCC TTG ATT GCT GAC ACC CCA TAC CAC CTG TAT CCT GCC TAC GGC TGG GTT ATG TTT GTA GCT GTC TTC CTC
Ala Leu Ile Ala Asp Thr Pro Tyr His Leu Tyr Pro Ala Tyr Gly Trp Val Met Phe Val Ala Val Phe Leu-51

        162         171         180         189         198         207         216         225
TGG CTG GTA ACA ATT GTC TTC TTC ATC ATC TAC CTG TTT CAA CTG CAC ATG AAG TTG TAT ATG GTG CCC TGG
Trp Leu Val Thr Ile Val Phe Phe Ile Ile Tyr Leu Phe Gln Leu His Met Lys Leu Tyr Met Val Pro Trp-75

        234         243         252         261         270         279         288         297
CCG TTG GTG TTA CTG GTC TTC TTT GTT GCT GCC ACC GTC CTC TAT ATC ACC GCC TTT GTC GCC TGT GCG GCG
Pro Leu Val Leu Leu Val Phe Phe Val Ala Ala Thr Val Leu Tyr Ile Thr Ala Phe Val Ala Cys Ala Ala-99

        306         315         324         333         342         351         360         369
GCG GTC GAT CTG ACA TCC CTG AGG GGC TCC CGA CCG TAC AAC CAG CGC TCG GCT GCC TCT TTC TTT GCT TGT
Ala Val Asp Leu Thr Ser Leu Arg Gly Ser Arg Pro Tyr Asn Gln Arg Ser Ala Ala Ser Phe Phe Ala Cys-123

        378         387         396         405         414         423         432         441
CTG GTG ATG ATT GCC TAC GGA CTG AGT GCT TTC TTC AGC TTC CAG GCC TGG CGA GGA GTG GGC AGC AAC GCG
Leu Val Met Ile Ala Tyr Gly Leu Ser Ala Phe Phe Ser Phe Gln Ala Trp Arg Gly Val Gly Ser Asn Ala-147

        450         459         468         477         486         495         504         513
GCC ACG AGT CAG ATG GCT GGG GGC TAC TCT TAA GCC AGC TAT GCT GTG ACC TAA GCC ACG GCT GGG TCT TAG
Ala Thr Ser Gln Met Ala Gly Gly Tyr Ser-157

        522         531         540         549         558         567         576         585
CAC AGG GCC ACC CCC AAC CTT ACC AGG CTT AAG CTC TCC AAG CAC TCC TGA GGA ACA GGC CCA AGC TGG CAC

        594         603         612         621         630         639         648         657
AGA TGG ACA GCC AAG AGC CAG GAG CGG GGA CAT CCT CCT CAC CTC CCT TAT TCA GCA GAA GAT GAT GGG GTT

        666         675         684         693         702         711         720         729
GGG GGG CTC TTG TGG CTT TGC CTT GTC ATC AGA GAA CTT CAG TGT CCC AGG CTG GGC CAC CCT GCT CAC CAG

        738         747         756         765         774         783         792         801
CAC TAA ATG CAC TAA CAG ACC TTC AGC CTC TGA TCT GCC TAG TGT AAA CCT AAC AAG ACA ACA CAT GAT CTA

        810         819         828         837         846         855         864         873
CGT TCC AGA AGC TAA GCC CAC CCC CAG GCT CTT CTG GTC CCA CCT CCC CCT CCC CTA CCG CAC GCC AAC CCA

        882         891         900         909         918         927         936         945
TTG TCA CCT GTA GAG AGA CTT TTA TAG AAG AGT TGG GAT TTC GAG GAC TGG ACT ATT TCA GTT GGT TTC AAT

        954         963         972         981         990         999        1008        1017
AGT TTC CAA GAC AGC AAG CCT CAC CAT GTC CCC TCC CTC ACG TGG AAC TCT TTC TCT CCC TTT GGG AGA GAG

       1026        1035        1044        1053        1062        1071        1080        1089
CAT CCG TGC AGG GTG CTT CCC CCA GGC TGC TGA TTA GAC ACA GGG GAC TTC CAA GGA GGG AGA GAG GGA AGC

       1098        1107        1116        1125        1134        1143        1152        1161
AGG GCC TTG CCT GGT GAG TTG TCA GGG GGT TAA TGG CGA CTC TTA TTT GGA ATT ATT ATT TTT TAC AAT TTA

       1170        1179
AAT AAA AAT GGA TGT GTC CGG AAT TC 3'
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FIG. 2. **Nucleotide and deduced amino acid sequence of plasmolipin cDNA.** The nucleotide sequence was obtained as described under "Experimental Procedures," and the amino acid sequence was deduced starting from the first initiation codon (ATG/Met). The amino acid sequence is extended up to the termination codon, TAA. The nucleotide sequence is *numbered* starting with the first ATG; all up stream codons are given *negative numbers*. The *underlined* nucleotide sequences are the signal recognition and consensus sequences for polyadenylation as defined under "Results."

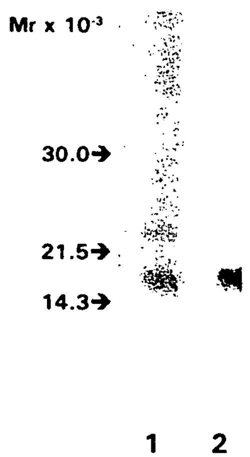

FIG. 3. **Digoxigenin detection of cysteine residues.** Proteins separated on 14% SDS gels under reducing conditions were transferred onto nitrocellulose membranes and either incubated with digoxigenin-labeled reagents selective for sulfhydryl groups (*lane 1*) or with anti-plasmolipin antibodies (*lane 2*) followed by the appropriate staining reactions (as described under "Experimental Procedures").

clones as plasmolipin. The size of the fusion protein was 130–135 kDa, which indicated an insert size of 15–20 kDa and suggested full-length plasmolipin was expressed.

Sequence Analysis of Plasmolipin cDNA—The nucleotide and the deduced amino acid sequences of the pPL1 cDNA clone are shown in Fig. 2. The nucleotide sequence contained an open reading frame, starting from the first ATG codon, encoding a 157-amino acid polypeptide of 17,400 Da. This value is very similar to the apparent molecular mass of plasmolipin (17–18 kDa) determined by SDS-gel electrophoresis (21). The initiation ATG codon had a flanking sequence that was homologous to the sequence of CC(A/G)CCATGG, which is conserved in eukaryotic initiation sites (32). At the 3′ end, the AATAAA sequence (Fig. 2, *underlined*) is a common polyadenylation signal, and the TGTGTCC sequence (Fig. 2, *underlined*) is similar to the consensus sequence of (C/T)GTGTTY(C/T)(C/T) often found between the poly(A) tail of the mRNA and the signal recognition site (33, 34).

The amino acid composition calculated from the deduced sequence confirmed earlier studies showing a high percentage of hydrophobic amino acids but also included cysteine and tryptophan residues, which were not observed in previous analyses of amino acid composition from acid hydrolysates. Since acid hydrolysis is not a reliable method for determination of all amino acids, the presence of cysteine in plasmolipin was demonstrated directly by using digoxigenin-linked maleimido labeling of sulfhydryl groups (Fig. 3). A periaxolemmal myelin proteolipid preparation, enriched in plasmolipin, was subjected to electrophoresis and transferred to nitrocellulose, and strips were probed either with digoxigenin-linked maleimido or with anti-plasmolipin (Fig. 3, *lanes 1* and *2*, respectively). Reactivity of the digoxigenin-reactive material co-migrated with immunoreactive plasmolipin, confirming the presence of cysteine in this protein. The higher molecular weight cysteine containing protein is probably the cysteine-rich PLP present in small amounts in these preparations (4).

The tryptophan content derived from the amino acid sequence confirmed previous physicochemical studies on plasmolipin that predicted the presence of at least 3 tryptophan residues and the localization of these residues in both hydrophobic and hydrophilic environments (2). The model for plasmolipin described below (Fig. 5), predicts 2 tryptophans in the bilayer and 3 tryptophans close to the phospholipid head groups near the membrane surface.

The composition of plasmolipin is characterized by the abundance of hydrophobic amino acids (Ala, Leu, Gly, Ile, Met, Phe, Trp, Tyr, and Val), which account for 72% of the total number of residues. The hydropathy plot (Fig. 4*A*) prepared by the method of Kyte and Doolittle (35) predicts four hydrophobic regions (I–IV) of 20–25 amino acids in length and predicts, using the method of Chou and Fasman (36), a secondary structure with high α-helical content (Fig. 4). Such a model corresponds to four transmembrane domains, which are separated by very short hydrophilic segments, except for a longer hydrophilic region between domains III and IV (Fig. 4*A*). The high level of predicted helical segments confirms earlier circular dichroism studies showing about 70% helical conformation when the protein is reconstituted in liposomes (2).

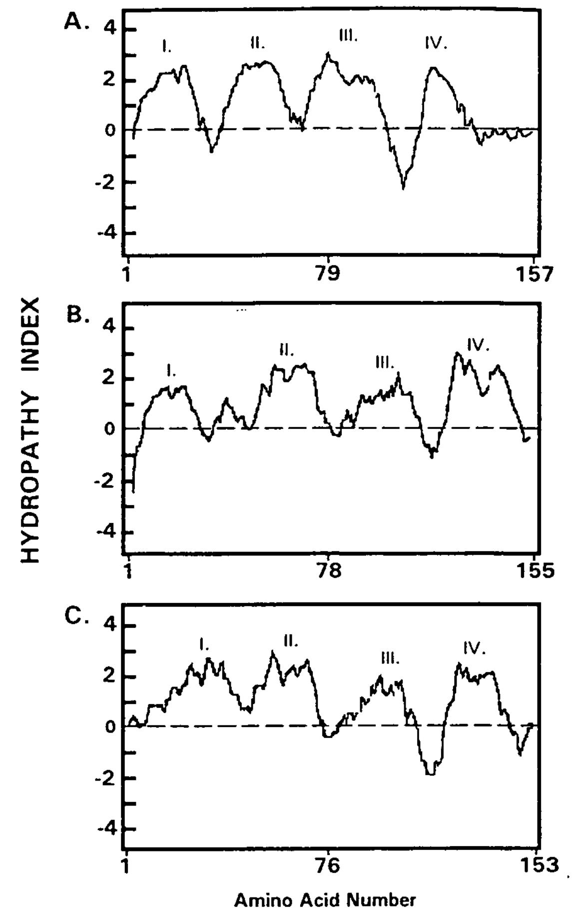

FIG. 4. **Hydropathy plot of plasmolipin and comparison with the MAL protein and the vacuolar H⁺-ATPase proteolipid.** The hydropathy plots were prepared according hydrophobic indices described by Kyte and Doolittle (35) and are based on the amino acid sequences of plasmolipin given in this report (*A*), MAL (37) (*B*), and the H⁺-ATPase proteolipid (15) (*C*).

Based on its mass and the hydropathy plots, plasmolipin (Fig. 4*A*) shows structural homology with other small hydrophobic membrane proteins with transport-related function; such as MAL, a 16.7-kDa human T cell receptor (37) (Fig. 4*B*), and the 16-kDa vesicular H⁺-ATPase of bovine chromaffin granules (15) (Fig. 4*C*). All three of these proteins have about 155 amino acids and, based on hydropathy plots, are composed of four transmembrane domains with a hydrophilic region between domains III and IV. Plasmolipin and these other two hydrophobic proteins therefore belong to a growing class of small hydrophobic proteins with four transmembrane segments, which also includes the myelin proteolipid proteins PLP and DM-20 (38).

The size and hydrophobicity of plasmolipin constrains the distribution of its transmembrane and extramembranous re-

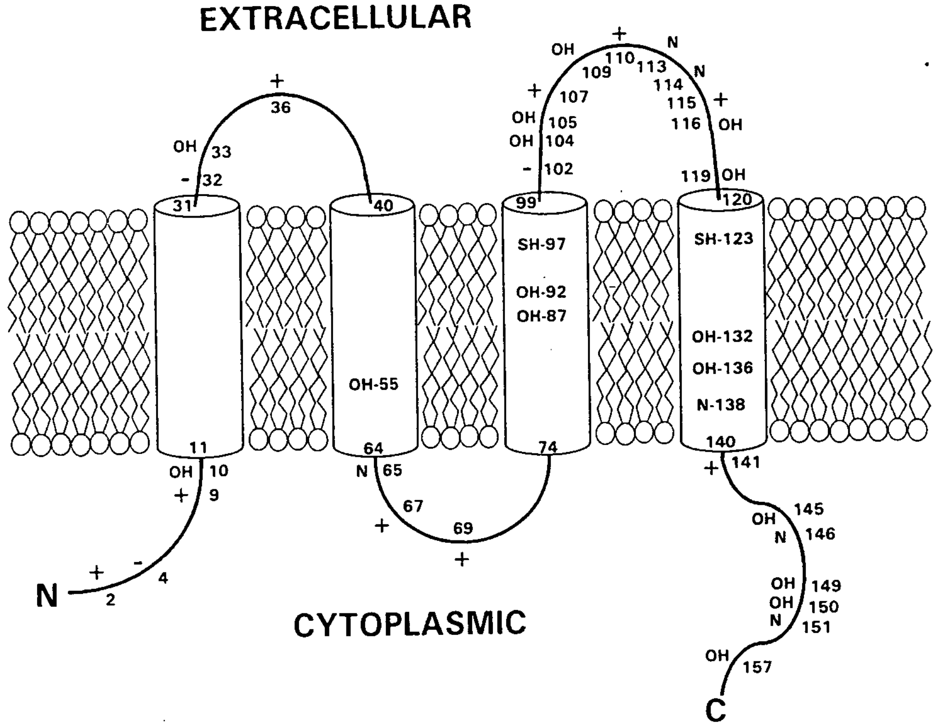

FIG. 5. **The proposed tertiary membrane structure of plasmolipin.** Based on distribution of hydrophobic amino acids in plasmolipin, as described in the hydropathy plot (Fig. 4), a tertiary structure of plasmolipin is proposed. *N* and *C* define the amino and carboxyl termini of the protein, respectively. The *numbers* in *parentheses* are residue numbers; (+) and (−) denote the respective positive and negative amino acid residues; *OH* and *SH* refer to the hydroxyl- and sulfhydryl-containing residues.

gions such that the orientation of the NH_2 and COOH termini both face the cytoplasm (Fig. 5). This is consistent with the known structure of many transport proteins (39) and the observation that channel-forming proteins cross the membrane an even number of times, placing both ends of the protein on the same membrane face (40). The model of plasmolipin shows that transmembrane segments III and IV contain hydroxyl groups and may indicate the structure of an aqueous channel. This model assigns cysteines 97 and 123 in domains III and IV, respectively, close to the external surface of such a channel where oxidation of these sulfhydryls could alter physiologic function. This structure also predicts tryptophans 75 and 140 are near the membrane-H_2O interface; previous structural analyses of transport proteins have predicted that tryptophan moieties in such amphipathic environments are important determinants of hydrophobic interactions (41). The prolines placed in the extramembranous regions between membrane domains I and II and between III and IV could facilitate bending of the external regions and allow a compaction of membrane domains.

Although some amino acid sequence homology exists between plasmolipin and the T cell receptor MAL protein (37), particularly in the second transmembrane domain (Fig. 6*B*), plasmolipin shows little overall primary sequence homology to any other protein in the GenBank and Protein Identification Resource (PIR) data bases. Nevertheless, each of the plasmolipin's transmembrane domains is similar to a variety of membrane transport proteins, *i.e.* the level of identity is over 20%, considered to be the minimum for the basis of homology (42). In the NH_2-terminal region (Fig. 6*A*), which includes transmembrane segment I, four examples of homology are given, including the bacterial glutamate/proton symport and gluconate permease (43), mammalian glucose transporter (44), and Na^+/H^+-antiporter (45). A series of more hydrophilic proteins such as human cadherin (46) and the yeast G_0/G_1 switch (47) protein also show with homology in this region. Since this is the only hydrophobic segment in these latter proteins, it suggests transmembrane segment I of plasmolipin may correspond to a membrane attachment motif. Within the second transmembrane domain (Fig. 6*B*), the only protein with significant homology, is the T cell receptor MAL protein. The third transmembrane domain (Fig. 6*C*), which we have proposed may participate in the formation of an aqueous channel, has homology with several groups of ion transport proteins. These include branched chain amino acid transport (48), glucose transport (49), H^+/K^+ exchange (50), and a rat K^+ channel (51).

Northern blot analysis of RNA prepared from various tissues confirmed that plasmolipin RNA was restricted to brain and kidney (Fig. 7). The mRNA obtained from brain and kidney was 1.7 kb; since plasmolipin is composed of 157 amino acids and the coding region is <0.5 kb, there are apparently relatively large untranslated segments. Previous biochemical studies indicated that plasmolipin exists as two subunits of similar composition, which differ by only 1 kDa. The resolution of the Northern blots was insufficient to demonstrate heterogeneity in mRNA that could correspond to such a small difference in protein mass.

Previous studies also indicated that in the brain the expression of plasmolipin is restricted to oligodendrocytes and is correlated with myelination (3, 5, 31). We therefore examined the expression of plasmolipin mRNA in oligodendrocytes over 4 weeks in culture (Fig. 8). The level of mRNA increased with time, correlating well with the changes in the level of plasmolipin protein (31).

Southern blots were used to analyze the structure of the plasmolipin gene using the PL1 probe. Genomic DNA was digested with *Xba*I, *Stu*I, and *Eco*RI (Fig. 9, *lanes 1–3*). Based upon the plasmolipin cDNA sequence, these enzymes should cleave at 0, 1, and 0 sites, respectively, and should therefore theoretically yield 1, 2, and 1 fragments. If any one of these enzymes yields the theoretical number of fragments, it is a strong indication that there is one plasmolipin gene. The data with *Xba*I and *Stu*I, where one and two fragments were obtained (*lanes 1* and *2*, respectively), are consistent with the presence of a single plasmolipin gene. The presence of an additional fragment with *Eco*RI (*lane 3*) may have resulted from a restriction site within an intron or a site outside of the coding region.

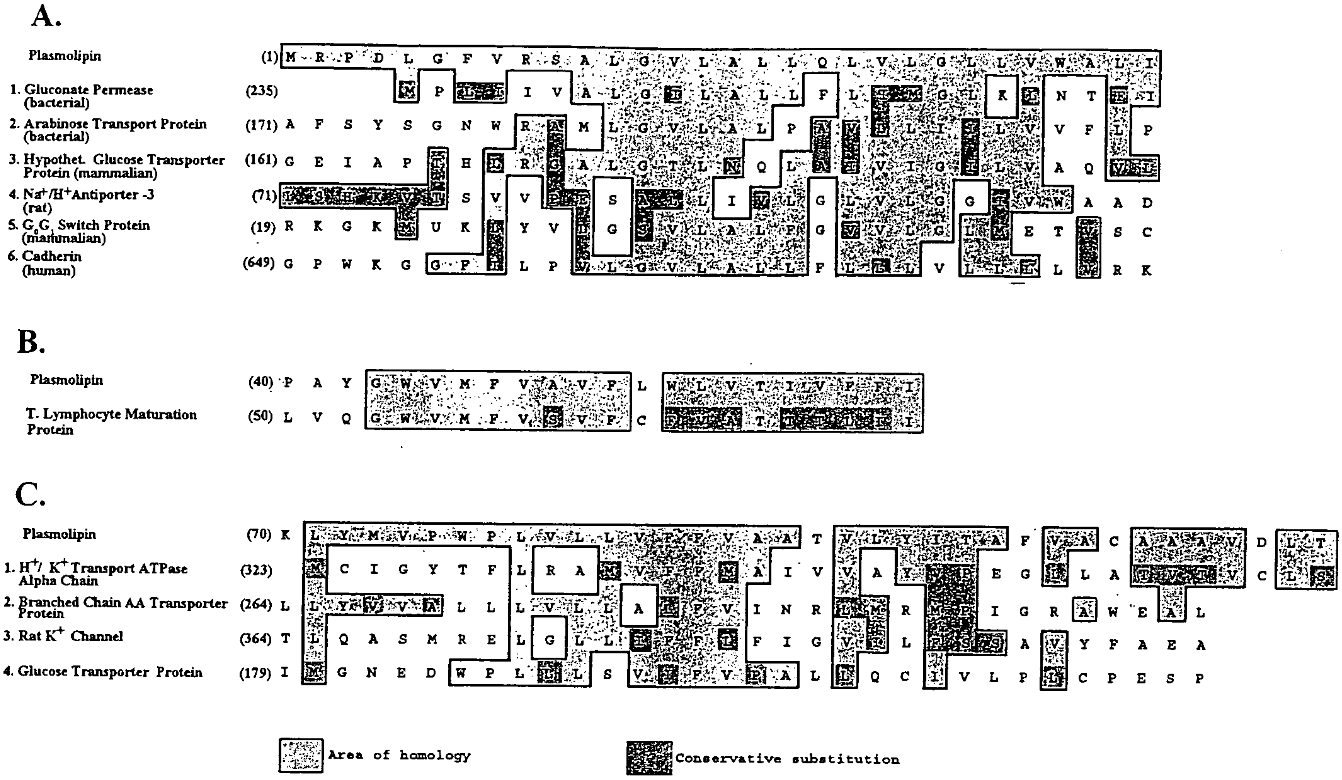

FIG. 6. **Analysis of plasmolipin homology.** Three regions of plasmolipin, which correspond approximately with transmembrane domains I–III (*A–C*), have been identified as having some homology with other membrane proteins in the PIR and SwissProt data bases using the Hitachi DNASIS search program. The proteins were selected only from those with the highest degree of homology. The *number* in *parentheses* next to each protein is the NH_2-terminal residue of the sequence given. The sequences of the proteins are as described in the reference noted in the text. *Lightly shaded* residues represent identical residues, and the *darker shaded* ones represent conservative substitutions.

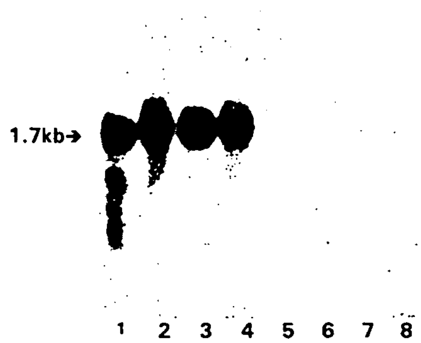

FIG. 7. **Tissue specificity of plasmolipin mRNA.** Northern blots analysis was carried out with 30 μg of total RNA prepared from different tissues, resolved on 1.2% agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled plasmolipin DNA (pPL1 probe). *Lanes 1–8* represent RNA from whole brain, kidney, cerebrum, cerebellum, liver, spleen, muscle, and adrenal gland, respectively. The size of the mRNA was estimated from the position of the 18 and 28 S rRNA and control hybridization with actin (23).

DISCUSSION

The identity of the cDNA isolated in this study as the one coding for plasmolipin has been confirmed at several levels. The fusion proteins obtained from the putative plasmolipin clones reacted specifically with two independent antibodies prepared against plasmolipin. The distribution of the mRNA, like the protein, was restricted to kidney and brain, and the changes in plasmolipin mRNA levels during oligodendroglial development followed the same pattern as the protein (31). In addition, the deduced size and structure of the cloned protein confirm earlier biochemical and biophysical studies of the purified plasmolipin (2, 21).

The presence of two similar, but not identical, plasmolipin subunits (1, 2) can result from several different mechanisms. Previous studies by *in vitro* translation experiments have indicated that the two subunits are not formed as a result of post-translational (proteolytic) processing (21). The presence of one DNA fragment on Southern blots after digestion with two restriction enzymes with no cleavage site within plasmolipin cDNA indicates that plasmolipin is encoded by a single gene. Therefore, the most plausible mechanism for the formation of two plasmolipin subunits is the presence of two transcripts, produced by alternative splicing, which could be resolved by Northern blots. Alternative splicing is a common mechanism that generates isoforms at the level of distinct transcripts, as is the case with myelin basic protein (52) and tau protein (53).

Proteolipids represent a distinct group of hydrophobic membrane proteins characterized in part by their solubility in organic solvents and their capacity to assume conformations compatible with solubility in water (2, 17) This amphipathic property does not appear to reside in any specific set of amino acid sequences, since several types of proteolipids can be defined which have little or no sequence homology with each other. The proteolipids of the mitochondrial, chloroplast, and vacuolar H^+ pumps represent one class with the latter arising from a gene duplication of the former (15). The cysteine-rich myelin proteolipids (PLP and DM-20) are another class of distinct proteolipids (16). These proteins, along with plasmolipin, do share one physical characteristic, which is a high number of transmembrane domains relative to their size. The T cell receptor-MAL protein (37) shares the high hydrophobic index and hydropathic pattern with plasmolipin and may therefore prove to be a proteolipid protein.

Proteolipids are associated with ion channel or transport systems (18–20). Plasmolipin and PLP have been shown to

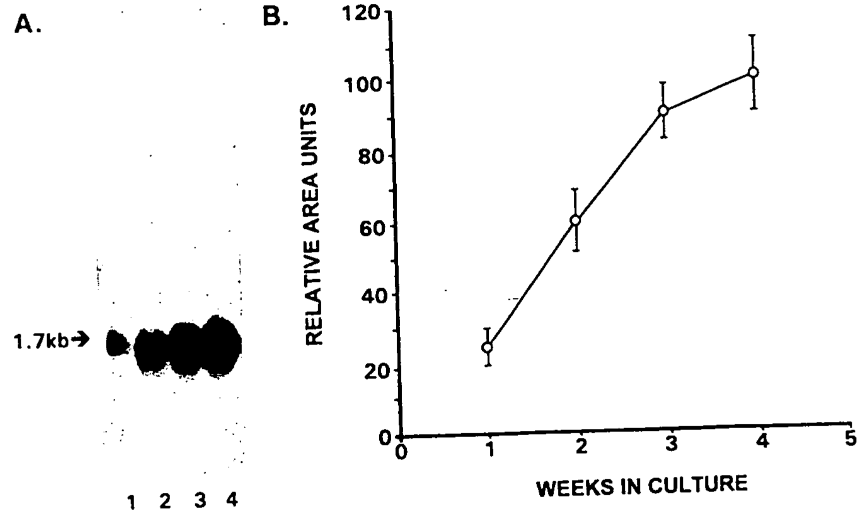

FIG. 8. **Expression of plasmolipin mRNA in developing oligodendrocytes.** Total RNA was prepared from oligodendrocyte-enriched cultures and analyzed by Northern blot. Equal amounts of RNA was loaded and resolved on a 1.2% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled plasmolipin probe. The blots (*A*) were scanned as described previously (26), and the integrated density values of the bands were plotted (*B*).

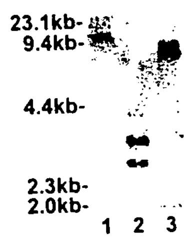

FIG. 9. **Restriction enzyme analysis of plasmolipin cDNA.** Genomic DNA was digested with *Xba*I, *Stu*I, and *Eco*RI (*lanes 1–3*, respectively), separated on agarose gels, blotted onto nylon membranes and hybridized with radiolabeled plasmolipin DNA probes as described under "Experimental Procedures."

form channels in artificial bilayers (1, 54). The MAL protein has been shown to share sequence elements with K^+ channels and has a pattern of expression that correlates with that of T cell receptor-linked ion channels (37). The homology analysis carried out in this study indicates that plasmolipin shares some sequence elements not only with the MAL protein in segment II but with several other transport proteins in segments I and III (43, 44, 46–49). The placement of cysteines near the extracellular face of this channel could be confirmed by experiments with impermeant sulfhydryl reagents. The presence of cysteine residues near the opening of a channel may be an important basis for physiologic control through oxidative state.

The proposed model for the quaternary structure of a plasmolipin channel consists of a hexamer of plasmolipin molecules arranged as a trimer each comprised of two plasmolipin subunits. This view is based upon two previous studies (1, 2). Plasmolipin-induced K^+ channels require a minimum oligomer of three. Physical chemical studies showed that plasmolipin has two observable oligomeric states, a dimer in non-polar solvent and a hexamer in aqueous environments suggesting the structure may be a trimer with each unit consisting of two plasmolipin subunits. This model portrays transmembrane segments III and IV as walls of a channel, a speculation consistent with the presence of hydroxyl groups in these domains. The compaction of the four transmembrane segments, depicted in this model, would be facilitated by the presence of prolines in the extramembranous sequences. This may be an important feature of proteolipid structure and function in general, since mutations involving proline residues of PLP have been demonstrated to be deleterious (55). Although we do not know what promotes hexamer formation of plasmolipin, we postulate that conditions which expose bilayer-associated hydrophobic amino acids to the aqueous membrane surface will lead to its aggregation (4).

Plasmolipin mRNA increases progressively in oligodendrocytes with their maturation in culture. These data conform with previous studies showing a similar temporal profile for the levels of plasmolipin protein (31). These data suggest that the biological function of plasmolipin is integral to properties of mature oligodendroglia. Its putative role in ion transport suggests this function may be the protection of the myelin complex from edematous insult through fluid volume regulation. The limitation of plasmolipin to the mammalian nervous systems (7) indicates its function relates to the unique conditions present in the brains of higher vertebrates.

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Full-length Cloning, Expression and Cellular Localization of Rat Plasmolipin mRNA, a Proteolipid of PNS and CNS

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Abstract

We have isolated a 1.476 bp cDNA (NTII11) representing a transcript that is differentially expressed during sciatic nerve development and regeneration in the rat. Nucleotide sequence comparison indicates partial identity with a recently isolated plasmolipin cDNA. However, our clone extends the published sequence by 234 bp at the 5′ end and predicts a protein that contains an additional 25 amino acids at the N-terminus. The open reading frame of the NTII11 transcript encodes a 19.4 kDa protein with four putative transmembrane domains. Northern blot analyses revealed a tissue-specific expression of the plasmolipin transcript that is restricted to sciatic nerve, brain and kidney. The neural expression was confirmed by *in situ* hybridization, and cellular localization of plasmolipin mRNA was demonstrated in Schwann cells of the sciatic nerve and in glial cells of myelinated brain structures. The steady-state levels of plasmolipin mRNA were markedly altered (i) during development of the sciatic nerve and brain, (ii) after sciatic nerve injury, and (iii) in cultured Schwann cells maintained under different conditions of cell growth and arrest. Our data indicate a function of plasmolipin during myelination in the central as well as in the peripheral nervous system.

Introduction

Myelin is a multilamellar structure ensheathing large axons in the peripheral nervous system (PNS) and central nervous system (CNS). The elaboration of this highly specialized membrane structure is reflected by the expression of myelin-specific genes in Schwann cells (PNS) and in oligodendrocytes (CNS) (for a review see Lemke, 1993). These genes encode proteins that are involved in the induction of myelination and the initial deposition, compaction or maintenance of the myelin sheath.

Among these genes, an expanding group has been identified that encodes structurally related, hydrophobic myelin proteins with four putative transmembrane regions, such as proteolipid protein (Milner *et al.*, 1986), the 22 kDa peripheral myelin protein (PMP22) (Spreyer *et al.*, 1991; Welcher *et al.*, 1991), the gap junction protein connexin-32 (Kumar and Gilula, 1986), the myelin and lymphocyte protein rMAL (Schaeren-Wiemers *et al.*, 1995), and plasmolipin (Fischer and Sapirstein, 1994). Very interestingly, most of them are candidate genes for hereditary dysmyelinating neuropathies, like proteolipid protein in Pelizaeus–Merzbacher disease (Hudson *et al.*, 1989), PMP22 in Charcot–Marie–Tooth disease type 1A (Timmerman *et al.*, 1992) and hereditary neuropathy with liability to pressure palsies (Chance *et al.*, 1993), and connexin-32 in X-linked Charcot–Marie–Tooth disease (Bergoffen *et al.*, 1993). Thus, a crucial role for these four transmembrane proteins in myelination is apparent. The expression of some of these genes is reportedly not confined to the nervous system, but is also found in other tissues, including lung (PMP22), liver (connexin-32), spleen (rMAL) and kidney (plasmolipin). With the exception of connexin-32, the biological function of these proteins is still unknown.

The term plasmolipin was given to a protein doublet (MW_A 11.5–17 kDa; MW_B = 13.5–18 kDa) initially isolated from kidney plasma membranes (Tosteson and Sapirstein, 1981). These proteins were classified as proteolipids (Lees *et al.*, 1979) based on their solubility in organic solvents due to the high proportion of hydrophobic amino acids. Sapirstein and co-workers demonstrated expression of plasmolipin in the nervous system using an antiserum directed to this group of proteins. Immunostaining of cultured neurons from rat hindbrain (Shea *et al.*, 1986), synaptic plasma membranes (Fischer and Sapirstein, 1986) and coated vesicles of bovine grey matter (Sapirstein *et al.*, 1988) suggests neuronal expression of plasmolipin. However, later publications described expression of these proteins in glial microsomes (Sapirstein *et al.*, 1988), oligodendrocytes (Shea *et al.*, 1986; Fischer *et al.*, 1991), myelin fractions (Cochary *et al.*, 1990) and coated vesicles of bovine white matter (Sapirstein *et al.*, 1992), which supports glial expression related to myelination.

In the present paper we report (i) the cloning of a plasmolipin cDNA from a sciatic nerve library, which encodes the entire protein, (ii) the tissue specificity of plasmolipin mRNA expression, (iii) the distinct cellular localization of plasmolipin mRNA in sciatic nerve

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and brain, (iv) the temporal pattern of changes in steady-state transcript levels in regenerating and non-regenerating nerve segments using two different lesion paradigms (crush and transection), (v) the expression of plasmolipin mRNA during development of sciatic nerve and brain, and (vi) the effect of forskolin and different conditions of cell growth and arrest on the steady-state concentration of this transcript in cultured Schwann cells.

Materials and methods

Animals and surgery

Adult Wistar rats (200–250 g) were anaesthetized with chloral hydrate (350 mg/kg body weight) administered intraperitoneally. Sciatic nerves were either crushed with jeweller's forceps or transected with a fine pair of scissors at upper thigh level (Müller *et al.*, 1986). In order to prevent spontaneous re-anastomosis, transected nerve stumps were tied with surgical silk. Prior to RNA preparation from nerve segments, the lesion zone (within 2–3 mm of the site of injury) was removed and discarded. All animal experiments were performed according to the guidelines of German animal rights law.

Schwann cell culture

Rat Schwann cells were prepared from sciatic nerves of Wistar rats aged 1–3 days, and purified by immunoselection as described by Brockes *et al.* (1979). A purity of at least 99% was obtained under these conditions, as confirmed by anti-Thy1.1 and anti-S100 immunostaining. Purified Schwann cell cultures were expanded in Dulbecco's modified Eagle medium (DMEM, Gibco–BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 100 μg/ml crude glial growth factor protein preparation and 2 μM forskolin (Sigma), for 4–6 weeks (Porter *et al.*, 1986). After growing to confluent or subconfluent cell layers, the cells were rinsed with DMEM/10% FCS and then cultured in the same medium, which was changed every 2 days, for a further 6 days. Then the cells were incubated for 72 h under the following conditions: (i) DMEM/10% FCS with 2 μM forskolin; (ii) DMEM/10% FCS without forskolin; (iii) DMEM/0.5% FCS with 2 μM forskolin.

Determination of cell proliferation

Cultures were incubated for 2 h in the presence of 100 μM 5-bromo-2′-deoxyuridine (BrdU). The cells were fixed in 4% paraformaldehyde/phosphate-buffered saline at room temperature for 5 min. DNA was denatured by treatment with 2 N HCl for 30 min at 37°C. After washing in Tris-buffered saline, the cells were incubated with mouse monoclonal antibody directed to BrdU (Boehringer Mannheim) at 4°C overnight. Detection of antibody was performed with the avidin-biotin peroxidase system (Vectastain, Vector Laboratories) according to the manufacturer's protocols.

Isolation of RNA

Total RNA was isolated from sciatic nerves by the acid–phenol–guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). The frozen tissues were homogenized with a Polytron (Brinkmann, Westbury, NY) at 25 000 r.p.m. for 2 × 45 s. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Sambrook *et al.*, 1989).

Preparation of the cDNA library

Poly(A)$^+$ RNA was isolated from adult rat sciatic nerve, 4.5 μg was primed with oligo(dT)$_{12\text{-}18}$ and cDNA was generated using the TimeSaver cDNA Synthesis Kit (Pharmacia–LKB, Piscataway, NJ). The cDNA was ligated into an *Eco*RI-predigested λZAPII phage (Stratagene, La Jolla, CA), and packaged into λ-phage particles using the GigapackII Packaging extract (Stratagene). Titration of the resulting cDNA library revealed a complexity of ~0.5 × 10^6.

DNA sequencing and computer analysis

Sequences of both strands were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7 Sequencing Kit (Pharmacia–LKB). Sequence data were compared with the EMBL nucleotide sequence data library and the SwissProt protein sequence databank using the programs FASTA (Pearson, 1990) and BLAST (Altschul *et al.*, 1990). Further analyses of the sequences were carried out with the PCGENE software package (Intelligenetics, Mountain View, CA) and with the PHDhtm program (Rost and Sander, 1994).

In vitro translation

Plasmolipin cDNA clones representing nucleotides 174–1476 (NTII11-2A; Fig. 1B) and nucleotides 233–1476 (NTII11-2; Fig. 1B) were linearized with *Xba*I or *Hin*dIII prior to the *in vitro* transcription in order to obtain sense- and antisense-oriented transcripts. Capped transcripts were synthesized from 2 μg of each linearized plasmid DNA in 100 μl transcription mix containing 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 50 U RNasin (Promega, Madison, WI), 500 μM rATP, rCTP, UTP, 50 μM rGTP, 1 mM m7G(5′)ppp(5′)G and 40 U T7 RNA polymerase. The reaction was performed at 37°C for 60 min. Template DNA was removed by digestion with 4 U RQ1 RNase-free DNase (Promega) at 37°C for 15 min, following extraction with phenol/chloroform and ethanol precipitation. RNA samples were recovered in RNase-free water at a concentration of 1 μg/μl. An aliquot of 2 μg was analysed for integrity of the transcripts by gel electrophoresis on a 3% agarose gel under denaturing conditions. Capped transcripts were translated *in vitro* using commercial rabbit reticulocyte lysates (Amersham). One microgram of each transcript or water as a control was heated for 15 min at 56°C, briefly chilled on ice and then incubated for 120 min at 30°C in 50 μl reaction mix containing 20 μl reticulocyte lysate, 40 μCi [^{35}S]methionine (1000 Ci/mmol), 1.5 mM magnesium acetate and 105 mM potassium chloride. Samples of 10 μl were adjusted to a final concentration of 4 M urea, 3% SDS and 2 mM dithiothreitol and subjected to gel electrophoresis on 12% SDS–PAGE gels under reducing conditions, processed for fluorography and subsequently exposed to X-ray films.

Northern blot analysis

Ten micrograms of total RNA was fractionated on 1.2% agarose gels containing 15% formaldehyde as described by Carmichael (1980) and then transferred to Nytran NY13N membranes (Schleicher and Schuell, Keene, NH) using standard protocols. The filters were subjected to ultraviolet cross-linking on a 302 nm transilluminator, prehybridized in 7% SDS, 0.5 M sodium phosphate (pH 7.0) and hybridized in the same solution containing 1–5 × 10^6 c.p.m./ml of a ^{32}P-labelled cDNA probe. A plasmolipin cDNA fragment representing nucleotides 372–1476 (NTII11-1, Fig. 1) was labelled with [^{32}P]dCTP using the random hexamer procedure (Feinberg and Vogelstein, 1984). Prior to exposure, the filters were washed in 2 × SSC/1% SDS at 60°C for 15 min and in 0.1 × SSC/1% SDS at 60°C for 15 min. Nytran filters were stained with methylene blue prior to hybridization (Sambrook *et al.*, 1989). After exposure to X-ray film (X-Omat AR, Kodak), the autoradiographic signals were quantified using a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

A

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   1                                   TGCCCAAACACCAGCACACTAGGGGTTCTTCACGA
  36  TCTCTATCCAGAGCAAACAGGCGAACCGACGGTTAAGGGGCGGGGCCTCTTCGAGGGGCG
  96  GGGCCTCGGGCGGAGCGGGGTCGGGCTTCGCGCACCTGTAGGTTCTGTCTAGGGATCCGC
 156  TGTCGCAGCCCCCGGAGCAGCGCCGGGAGCGCGTCCCGAGCGTCGTAGGCTCGCGATCGCT
 216  ATGGCGGAGTTCCCGTCGAAAGTGAGCACCAGGACCAGCAGCCCCGCGCAGGGCGTGGGA
      METAlaGluPheProSerLysValSerThrArgThrSerSerProAlaGlnGlyValGly  20
                              ▲
 276  GCATCTGTGTCTGCGATGCGACCGGACCTGGGCTTTGTGCGCTCCGCCCTTGGGGTGCTC
      AlaSerValSerAlaMetArgProAspLeuGlyPheValArgSerAlaLeuGlyValLeu  40

 336  GCGCTTCTGCAGCTGGTGCTGGGGCTGCTGGTGTGGGCCTTGATTGCTGACACCCCATAC
      AlaLeuLeuGlnLeuValLeuGlyLeuLeuValTrpAlaLeuIleAlaAspThrProTyr  60

 396  CACCTGTATCCTGCCTACGGCTGGGTTATGTTTGTAGCTGTCTTCCTCTGGCTGGTAACA
      HisLeuTyrProAlaTyrGlyTrpValMetPheValAlaValPheLeuTrpLeuValThr  80

 456  ATTGTCTTCTTCATCATCTACCTGTTTCAACTGCACATGAAGTTGTATATGGTGCCCTGG
      IleValPhePheIleIleTyrLeuPheGlnLeuHisMetLysLeuTyrMetValProTrp  100

 516  CCGTTGGTGTTACTGGTCTTCTTTGTTGCTGCCACCGTCCTCTATATCACCGCCTTTGTC
      ProLeuValLeuLeuValPhePheValAlaAlaThrValLeuTyrIleThrAlaPheVal  120

 576  GCCTGTGCGGCGGCGGTCGATCTGACATCCCTGAGGGGCTCCCGACCGTACAACCAGCGC
      AlaCysAlaAlaAlaValAspLeuThrSerLeuArgGlySerArgProTyrAsnGlnArg  140
                               ▲
 636  TCGGCTGCCTCTTTCTTTGCTTGTCTGGTGATGATTGCCTACGGACTGAGTGCTTTCTTC
      SerAlaAlaSerPhePheAlaCysLeuValMetIleAlaTyrGlyLeuSerAlaPhePhe  160

 696  AGCTTCCAGGCCTGGCGAGGAGTGGGCAGCAACGCGGCCACGAGTCAGATGGCTGGGGGC
      SerPheGlnAlaTrpArgGlyValGlySerAsnAlaAlaThrSerGlnMetAlaGlyGly  180

 756  TACTCTTAAGCCAGCTATGCTGTGACCTAAGCCACGGCTGGGTCTTAGCACAGGGCCACC
      TyrSer                                                        182
 816  CCCAACCTTACCAGGCTTAAGCTCTCCAAGCACTCCTGAGGAACAGGCCCAAGCTGGCAC
 876  AGATGGACAGCCAAGAGCCAGGAGCGGGGACATCCTCCTCACCTCCCTTATTCAGCAGAA
 936  GATGATGGGGTTGGGGGGCTCTTGTGGCTTTGCCTTGTCATCAGAGAACTTCAGTGTCCC
 996  AGGCTGGGCCACCCTGCTCACCAGCACTAAATGCACTAACAGACCTTCAGCCTCTGATCT
1056  GCCTAGTGTAAACCTAACAAGACAACACATGATCTACGTTCCAGAAGCTAAGCCCACCCC
1116  CAGGCTCTTCTGGACCCACCTCCCCCTCCCCTACCGCACGCCAACCCATTGTCACCTGTA
1176  GAGAGACTTTTATAGAAGAGTTGGGATTTCGAGGACTGGACTATTTCAGTTGGTTTCAAT
1236  AGTTTCCAAGACAGCAAGCCTCACCATGTCCCCTCCCTCACGTGGAACTCTTTCTCTCCC
1296  TTTGGGAGAGAGCATCCGTGCAGGGTGCTCCCCCCAGGCTGCTGATTAGACACAGGGGAC
1356  TTCCAAGGAGGGAGAGAGGGAAGCAGGGCCTTGCCTGGTGAGTTGTCAGGAGGTTAATGG
1416  CGACTCTTATTTGGAATTATTATTTTTTACAATTTAAATAAAAATGGATGTGTCTGTTTG
1476  @ poly (A)
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B

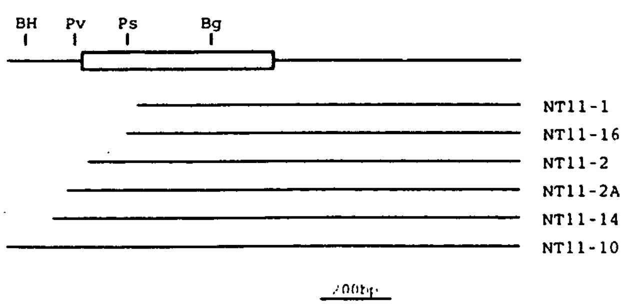

FIG. 1. (A) Nucleotide sequence of plasmolipin cDNA and deduced amino acid sequence. A continuous open reading frame extends from nucleotide 216 to nucleotide 761. The first start codon in the proximity of the 5′ end appears in the favourable nucleotide context (ATCGCT-ATG-G) of the consensus sequence (Kozak, 1991) for translation initiation. The 25 N-terminal amino acids extending the deduced sequence of Fischer and Sapirstein (1994) are shown in bold letters. Further differences in the nucleotide sequence with the cDNA published by Fischer and Sapirstein (1994) were found in the 3′ untranslated region (bold letters). Four hydrophobic amino acid stretches predicting putative transmembrane domains are underlined and two putative phosphorylation sites (Ser9, Ser130) are indicated by triangles. A dashed line marks the polyadenylation signal (AATAAA). (B) Schematic representation and restriction map of the plasmolipin cDNA. The cDNA clone contains a 5′ leader sequence of 215 nucleotides followed by 546 nucleotides coding for the protein (hatched box). The 3′ non-coding region extends for 714 nucleotides. Restriction sites: Bg, *Bgl*I; BH, *Bam*HI; Ps, *Pst*I; Pv, *Pvu*I. A schematic representation of the six NTII11 cDNA clones that differed only in the length of their 5′ regions is given below.

In situ hybridization

Different tissues were embedded in Tissue Tec II (Miles, Naperville, IL) frozen in methyl butane at −70°C and cut into 10 μm sections. The sections were fixed and subsequently acetylated and prehybridized at 55°C for 4 h according to Angerer *et al.* (1987).

Run-off transcripts from the 1.3 kb plasmolipin cDNA-clone NTII11–2A were prepared using digoxigenin-UTP and the DIG-RNA labelling kit from Boehringer Mannheim. After hybridization at 55°C overnight, RNase A treatment (10 μg/ml in 0.5 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8) was carried out at 37°C for 20 min. The sections were then washed three times in 2 × SSC at 50°C for 20 min and three times in 0.1 × SSC at 50°C for 20 min. Digoxigenin detection was carried out following the manufacturer's instructions (Boehringer Mannheim).

Results

Cloning and sequence analysis

In order to clone genes that are differentially regulated after sciatic nerve injury, we isolated a 1105 bp long cDNA clone (NTII11–1) from a cDNA library that was constructed with poly(A)$^+$ RNA derived from non-injured sciatic nerve and was subjected to differential colony screening (Spreyer *et al.*, 1991; Gillen *et al.*, 1995). Two rounds of rescreening yielded five additional independent clones (Fig. 1), the longest clone contained a cDNA of 1476 bp. The nucleotide sequence of this clone and the deduced amino acid sequence are shown in Figure 1A. Computer-assisted sequence comparison indicated identity to the very recently published cDNA sequence of the proteolipid plasmolipin (Fischer and Sapirstein, 1994) with the following important difference—our cDNA extends the published sequence by 234 bp at the 5′ end, and due to an ATG initiation site (nucleotide 216) that is 75 bp upstream of the previously published ATG (nucleotide 291) the present sequence predicts a protein that is 25 amino acids longer. Since the N-terminal extension is hydrophilic in sequence, the protein probably lacks a signal peptide.

The open reading frame encodes a 182 amino acid polypeptide of 19.4 kDa that contains four hydrophobic amino acid stretches

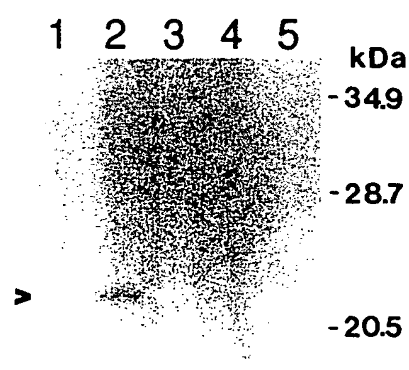

FIG. 2. Autoradiogram of plasmolipin *in vitro* translation. The analysis was performed with sense (lane 2) and antisense transcripts (lane 3) derived from clone NTII11–2A (nucleotides 174–1476), and with sense (lane 4) and antisense (lane 5) transcripts derived from clone NTII11–2 (nucleotides 233–1476). In lane 1 water was used as a negative control. The positions of the molecular weight markers are given at the right. Note that only the sense transcript derived from clone NTII1–2A is translated into a protein of 22 kDa (arrowhead).

(underlined in Fig. 1), as revealed by hydropathy index computation using SOAP (Klein *et al.*, 1985). Analysis with the neural network method PHDhtm (Rost and Sander, 1994) predicted four transmembrane helices with an accuracy of 95%. Computer analysis further revealed two potential phosphorylation sites at positions Ser9 and Ser130 (Figs 1A and 8).

A computer search of the Swissprot and PIR databases showed no significant overall homology to other proteins; only the second transmembrane domain revealed a 50% identity with the protein T-MAL, a maturation-associated protein of human T-lymphocytes (Alonso and Weissman, 1987), and with rMAL, the rat homologue of T-MAL (Schaeren-Wiemers *et al.*, 1995), respectively.

Northern hybridization of a [^{32}P]dCTP-labelled 1.1 kb fragment (extending from nucleotide 372 to 1476) of the plasmolipin cDNA to total RNA isolated from mature non-injured rat sciatic nerve detected a transcript of ~1.7 kb (Fig. 3A), in agreement with the results of Fischer and Sapirstein (1994). Taking into account that poly(A) tails usually extend for ~150–200 residues, the cDNA clone shown in Figure 1 is nearly full-length.

In vitro translation of plasmolipin

In order to determine the correct translation initiation site, *in vitro* translation was performed using two plasmolipin transcripts of different length. Sense and antisense *in vitro* transcripts were derived from (i) the clone NTIII1–2A, extending from nucleotide 174 to 1476, which contains both putative translation initiation sites; and (ii) the clone NTII11–2, extending from nucleotide 233 to 1476, therefore containing only the second ATG at nucleotide 291. *In vitro* translation yielded a protein of 22 kDa only when the sense transcript of clone NTII11–2A was used (Fig. 2, lane 2). No protein was found with the sense transcript derived from clone NTII11–2 (Fig. 2, lane 4). The specificity of the reaction was demonstrated by using water (lane 1) and the antisense transcripts of both cDNAs (lanes 3 and 5) as negative controls respectively.

Tissue-specific expression of plasmolipin mRNA

Northern blot analysis using total RNA prepared from various adult rat tissues revealed the highest expression in sciatic nerve, followed by kidney and brain; no signal was found in lung, heart, muscle, testis, liver, thymus and spleen (Fig. 3A). Densitometric evaluation of hybridization signals showed that the plasmolipin transcript is 2- and 7-fold more abundant in peripheral nerve than in kidney and brain respectively.

Lesion-induced changes of plasmolipin mRNA levels in sciatic nerve

We have further analysed temporal changes in the relative abundance of plasmolipin mRNA in rat sciatic nerve by Northern blots following

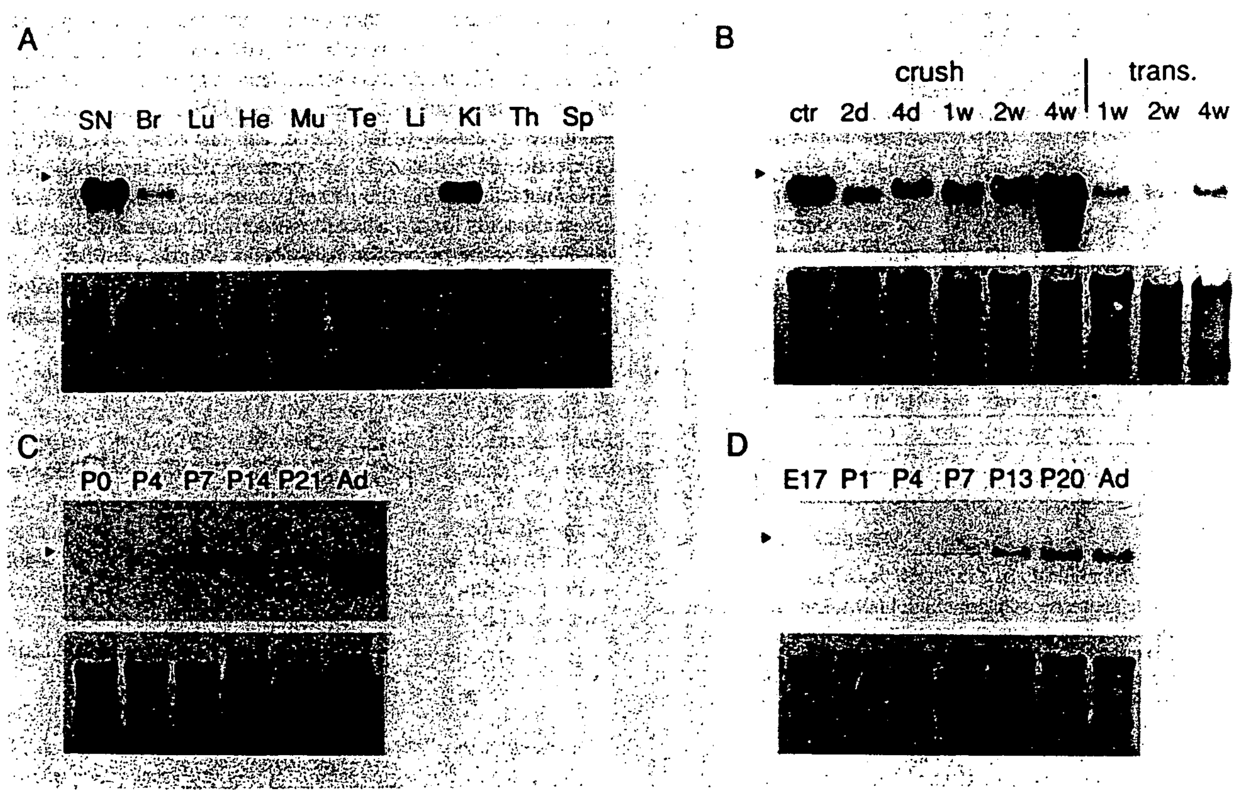

FIG. 3. Northern blots showing the tissue-specific (A), lesion-specific (B) and developmental (C, D) expression of the plasmolipin transcript in rat. (A) Ten micrograms of total RNA derived from sciatic nerve (SN), brain (Br), lung (Lu), heart (He), skeletal muscle (Mu), testis (Te), liver (Li), kidney (Ki), thymus (Th) and spleen (Sp) was hybridized with a [^{32}P]dCTP-labelled 1.1 kb fragment of plasmolipin cDNA. (B) Nerves were either crushed or transected and distal segments were used to prepare total RNA from each time point indicated (d, days; w, weeks). Ten micrograms of RNA was hybridized with the cDNA-fragment described in A. ctr, control non-injured nerve. (C) Northern blot analysis of developing sciatic nerve. Ten micrograms of total RNA derived from sciatic nerves of Wistar rats at postnatal days 0, 4, 7, 14 and 21 (P0–21) and adult animals (Ad) were hybridized as described in A. Note that at postnatal day 21 the weaker signal is due to a smaller amount of RNA, as shown by methylene blue-stained filter in the lower trace. (D) Northern blot analysis of developing rat brain. Ten micrograms of total RNA derived from entire brain of adult rats (Ad), embryonic rats at day 17 (E17), and Wistar rats at postnatal days 1, 4, 7, 13 and 20 (P1–20) were hybridized as described in A. For details see Materials and methods. The arrowhead indicates the position of the 18S ribosomal RNA (1.87 kb). Lower traces show methylene blue staining of the Nytran filter prior to hybridization.

two types of lesions: (i) crush injury, which leads to Wallerian degeneration in the distal nerve segment prior to regeneration of axons from the proximal stump into the distal segment; and (ii) transection of nerve and permanent separation of both stumps by ligation in order to prevent axonal growth into the degenerated distal stump. In the distal stump of crushed and transected nerve the transcript levels declined to very low levels within 2 days and 1 week after lesion respectively (Fig. 3B). During the second week after a crush injury the transcript levels began to rise again, reaching a level 4 weeks after lesion that was even higher than in the non-injured control sciatic nerve. In contrast, in the distal stump of the transected sciatic nerve, which was prevented from regeneration, the mRNA steady-state level remained low for at least 4 weeks after axotomy.

Developmental expression of plasmolipin mRNA in sciatic nerve and brain

Sciatic nerve

Northern blot analyses were carried out using total RNA isolated from sciatic nerves of rats from the first postnatal day (P1) throughout adulthood. Expression of the plasmolipin transcript could first be detected at day P4, increased to a maximum at day P14 and then declined to a moderate level in adulthood (Fig. 3C).

Brain

Using Northern blot analysis we further investigated the temporal expression of plasmolipin mRNA during development of the brain from embryonic day 17 and throughout adulthood. Following the onset of expression at P1, the transcript level steadily increased to a maximum at P20 and then decreased slightly to adulthood (Fig. 3D).

Cellular localization of plasmolipin mRNA by in situ hybridization

In situ hybridization of a digoxigenin–UTP-labelled antisense RNA probe to longitudinal sections of non-injured mature rat sciatic nerve revealed signals associated with myelinated nerve fibres (Fig. 4A, C, D), but which were not seen in control experiments using a digoxigenin–UTP-labelled sense RNA probe (Fig. 4B). Higher magnification revealed the longitudinal bipolar morphology of labelled Schwann cells (Fig. 4 A, D).

In situ hybridization of the same probe to frontal sections of rat brain, derived from 21-day-old animals, labelled cells in myelinated brain regions (Figs 5 and 6). On the other hand, no signals were detected using a digoxigenin–UTP-labelled sense RNA probe (Fig. 5C). The most intense labelling was found in the corpus callosum (Fig. 5A, B), the cortical white matter (Fig. 6A) and in the optic

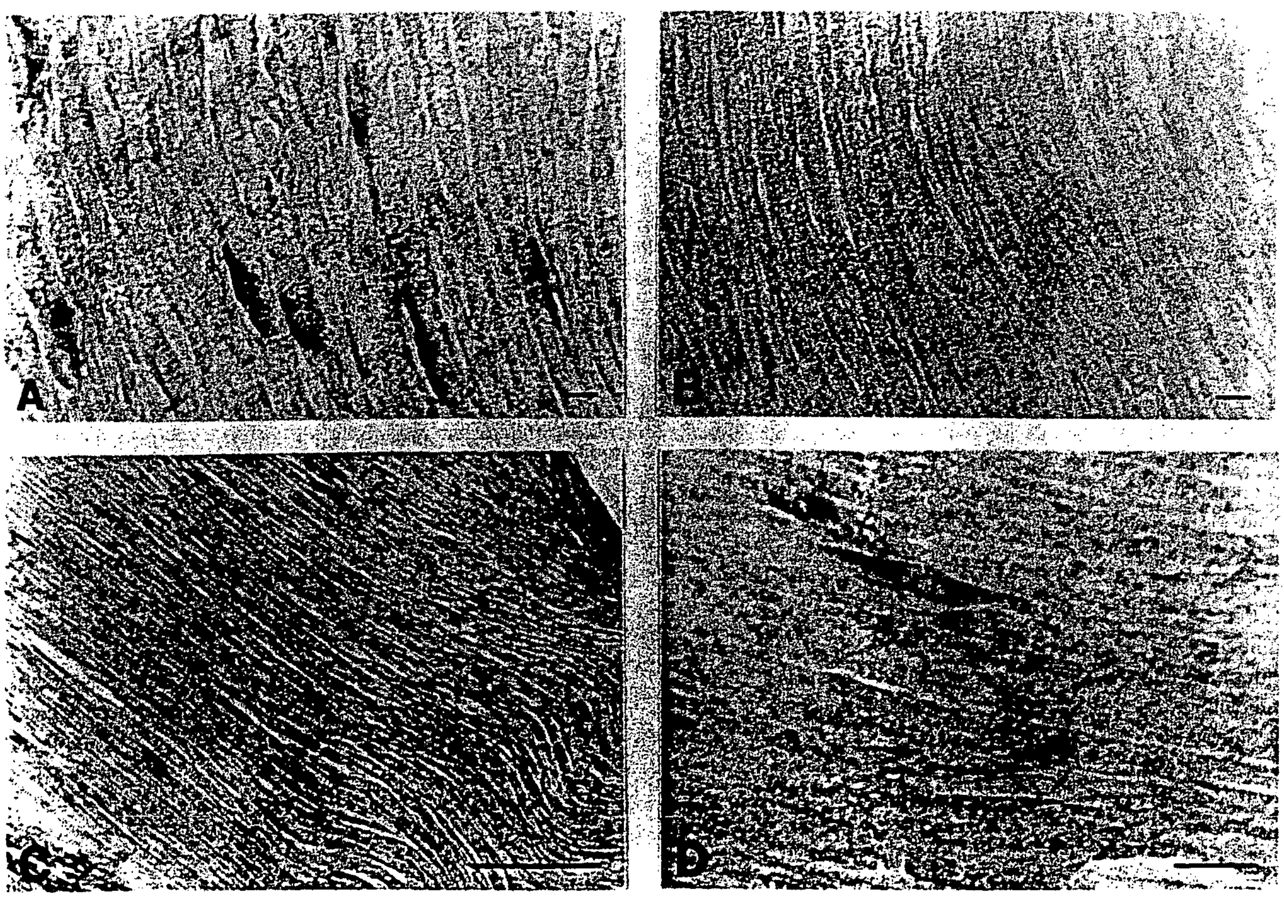

FIG. 4. Cellular localization of the plasmolipin transcript in longitudinal sections of adult rat sciatic nerve by *in situ* hybridization. (A, C, D) *In situ* hybridization with a digoxigenin–UTP labelled antisense RNA. (B) *In situ* hybridization with a digoxigenin–UTP-labelled sense RNA probe to a parallel section (negative control). Bar in A, B, D = 10 μm, bar in C = 100 μm.

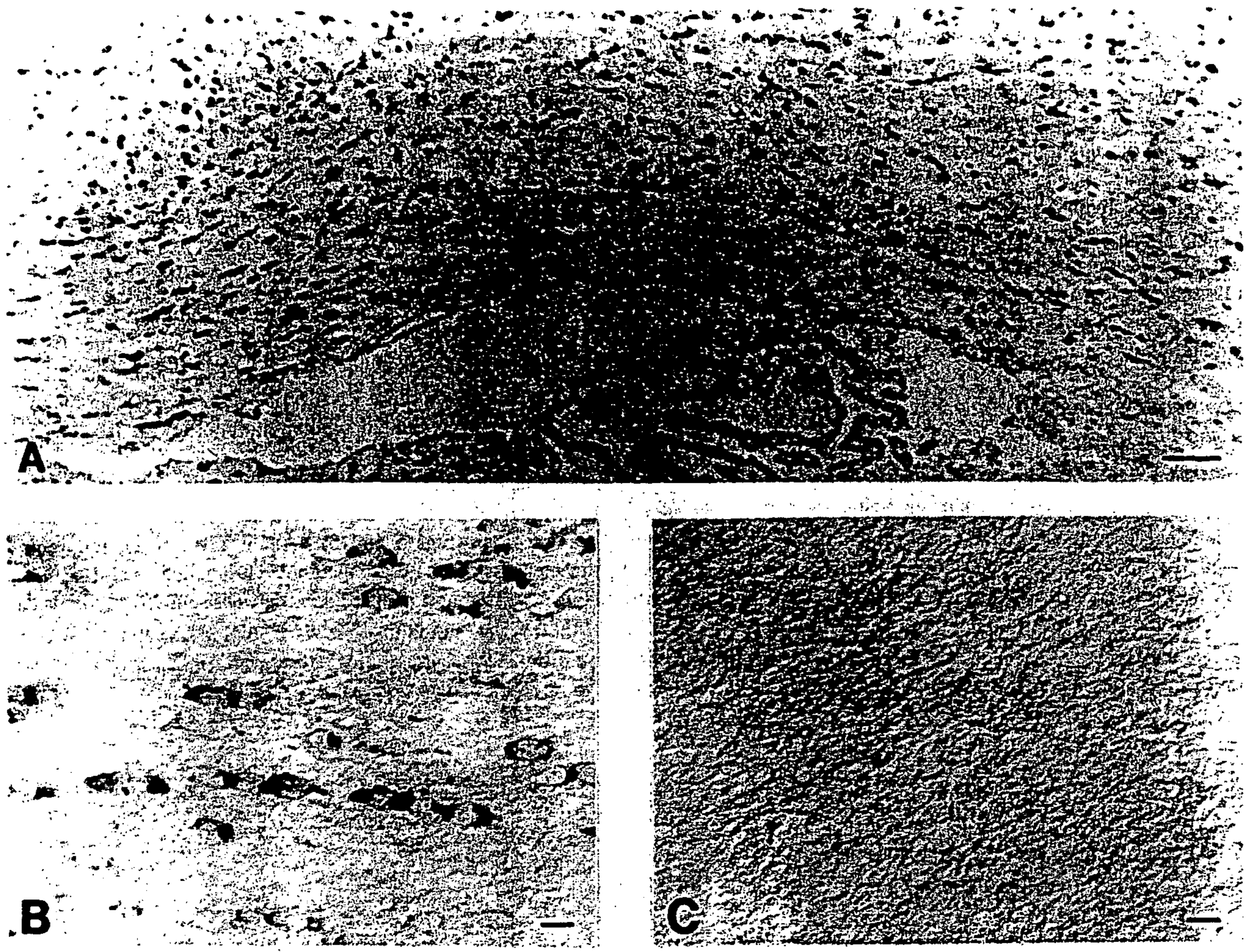

FIG. 5. Cellular localization of the plasmolipin transcript in corpus callosum of rat brain by *in situ* hybridization. *In situ* hybridization was performed with a 1.3 kb digoxigenin–UTP-labelled antisense RNA probe (A, B) or with a digoxigenin–UTP-labelled sense RNA probe (C) as negative control to a parallel section. Bar in A = 100 μm, bar in B, C = 10 μm.

nerve (Fig. 6D). In the corpus callosum, the labelled cells showed a string of pearl-like orientation. In the hippocampus, most signals were seen in the stratum radiatum and stratum oriens (Fig. 6B). No signals could be observed in the meninges, cortex layer I (Fig. 6A) and in the plexus chorioideus (Fig. 6C).

Expression of plasmolipin mRNA in cultured Schwann cells

Schwann cells from neonatal rat sciatic nerve were cultured to determine the effect of cell density, proliferation, serum and forskolin concentration on the relative abundance of plasmolipin mRNA. In subconfluent, proliferating Schwann cell cultures lacking forskolin no plasmolipin mRNA could be detected (Fig. 7A, lane 3–), whereas in sibling cultures containing 2 μM forskolin for 72 h very low levels of the transcript were observed (Fig. 7A, lane 3+). The proliferative state of these cells was demonstrated by BrdU incorporation (Fig. 7B, lanes 3+ and 3–). In contrast, confluent non-proliferating Schwann cells expressed significantly higher levels of plasmolipin mRNA (Fig. 7, lanes 1+, 1–, 2+ and 2–). It is interesting to note that both (i) a decrease in serum (FCS) concentration from 10 to 0.5% (Fig. 7A, lane 1+ versus 2+ and lane 1– versus 2–) and (ii) the addition of 2 μM forskolin, a specific activator of adenylate cyclase (Fig. 7A, lane 1+ versus 1– and lane 2+ versus 2–) up-regulated plasmolipin mRNA in Schwann cells. Both stimulatory effects seem to be additive.

Discussion

We have cloned a cDNA complementary to a 1.7 kb transcript that is differentially expressed in Schwann cells during peripheral nerve regeneration in rats. Sequence comparison reveals identity with the recently cloned plasmolipin cDNA (Fischer and Sapirstein, 1994), but due to a longer open reading frame our sequence codes for a protein extending by 25 amino acids at the N-terminus. It is likely that the most 5′-ATG at nucleotide 216 is the correct initiation site, because (i) it appears to be the first ATG, located 147 nucleotides downstream of an in-frame stop codon (nucleotide 69), and (ii) the region around this ATG is highly homologous to the Kozak consensus sequence GCC(A/G)CCATGG (Kozak, 1991). Furthermore, *in vitro* translation analysis (Fig. 2) revealed that the first ATG at nucleotide 216 is used as the plasmolipin start codon, yielding a protein of 22 kDa.

This additional amino acid sequence contains a putative phosphorylation site (Ser9) and, as a hydrophilic sequence, it points to the absence of a signal peptide. The deduced protein contains four

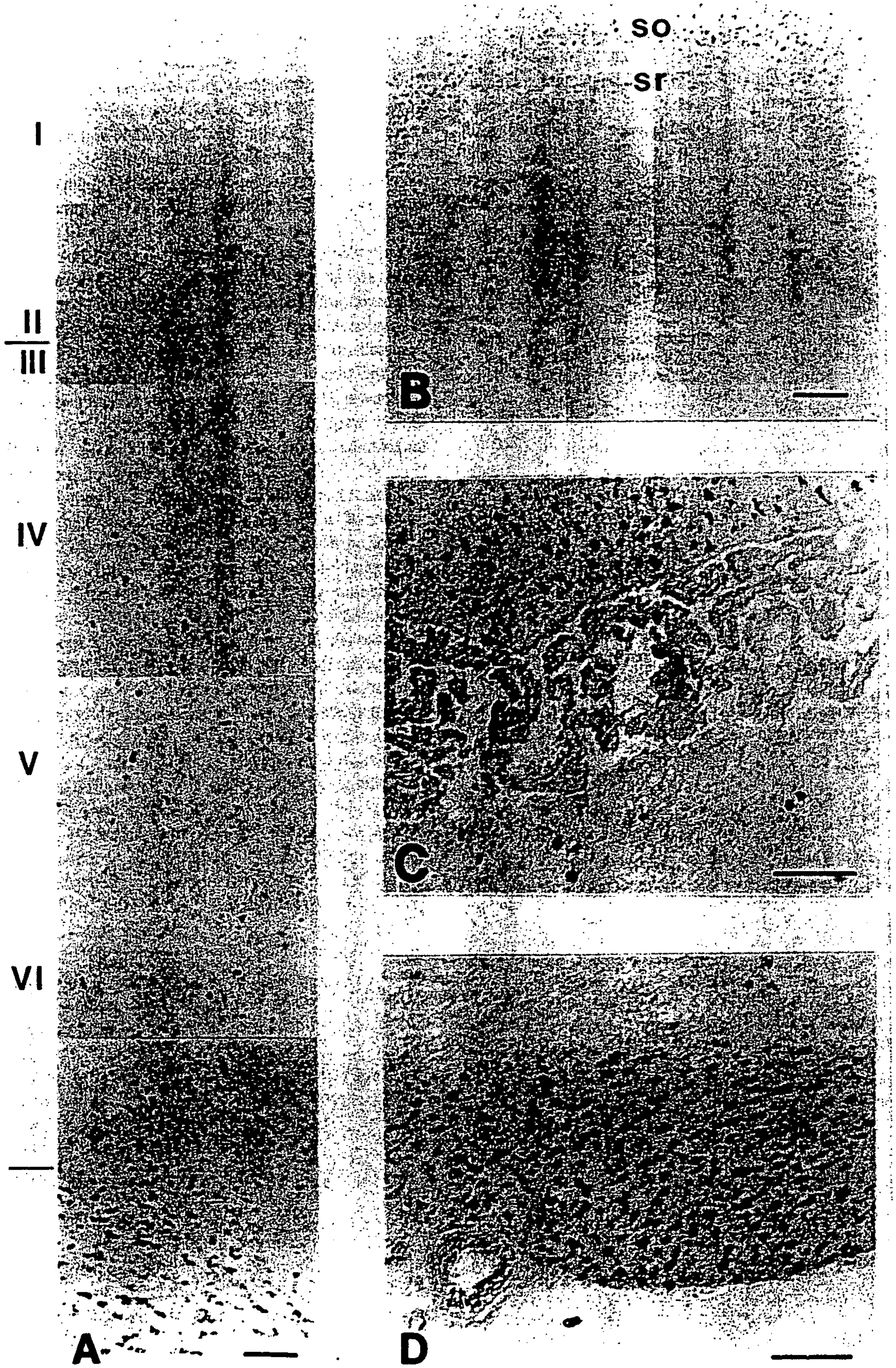

FIG. 6. *In situ* hybridization showing the cellular localization of the plasmolipin transcript in (A) different cortex layers, (B) hippocampus, (C) plexus chorioideus and (D) optic nerve. I–VI, first to sixth cortex layers; sr, stratum radiatum; so, stratum oriens. Bar = 100 μm.

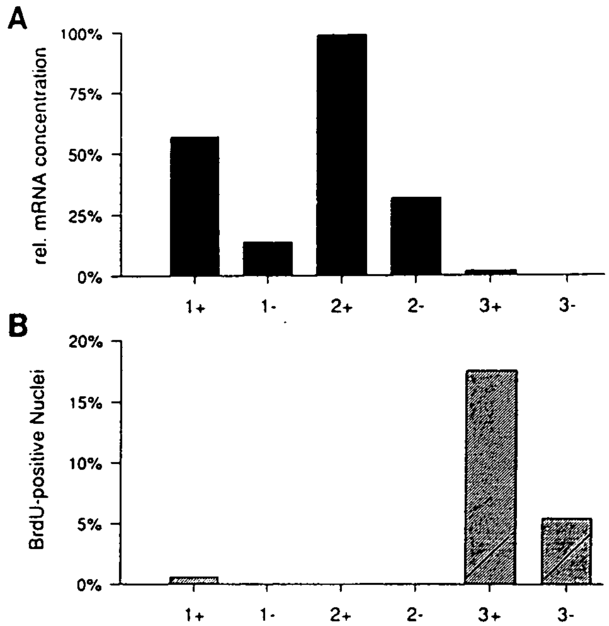

FIG. 7. Expression of plasmolipin mRNA under different growth conditions in cultured rat Schwann cells. (A) Densitometric evaluation of a Northern blot analysis of confluent and subconfluent Schwann cell layers incubated for 72 h in the presence (+) or absence (–) of 2 μM forskolin under the following conditions: (1) confluent cells in DMEM containing 10% FCS; (2) confluent cells in DMEM containing 0.5% FCS; (3) subconfluent cells in DMEM containing 10% FCS. (B) Analysis of cell proliferation by incorporation of BrdU into cells undergoing DNA synthesis. Immunocytochemical detection of incorporated BrdU was performed after 2 h incubation of cells in 100 μM BrdU. For details see Materials and methods.

hydrophobic domains that are putative membrane-spanning regions. Interestingly, other transmembrane myelin proteins, such as connexin-32, rMAL, PMP22 and proteolipid protein, also lack a signal peptide. The structural homology of plasmolipin with other transmembrane proteins—like T-MAL (Alonso and Weissman, 1987), rMAL (Schaeren-Wiemers *et al.*, 1995), PMP22 (Spreyer *et al.*, 1991) and the proteolipid subunit of the vacuolar H^+-ATPase (Mandel *et al.*, 1988)—supports the topological model shown in Figure 8. This model extends the protein structure previously predicted by Fischer and Sapirstein (1994).

The steady-state level of plasmolipin mRNA is highest in peripheral nerve followed by kidney and brain, whereas plasmolipin is not expressed in other non-neural tissues. The mRNA expression in kidney and brain has previously been reported by Fischer and Sapirstein (1994).

In the sciatic nerve the plasmolipin mRNA expression is confined to Schwann cells, as demonstrated by *in situ* hybridization. The identification of these cells is based upon (i) their typical bipolar longitudinal morphology and association with the nerve fibres (Fig. 4C), (ii) S100 immunoreactivity of these cells in additional sections of the same nerve (data not shown), and (iii) the large number of these labelled cells.

There are different lines of evidence suggesting that the mRNA expression of plasmolipin in the brain is restricted to myelinating oligodendrocytes: (i) *in situ* hybridization signals were found only in myelinated brain structures, with the highest abundance in the corpus callosum (Fig. 5A) and optic nerve (Fig. 6D), (ii) the string of pearls-like orientation of the labelled cells in the corpus callosum (Fig. 5A, B) is typical of oligodendrocytes and has been described previously (so-called *Kernreihen*; Fleischhauer and Wartenburg, 1967; Berger and Frotscher, 1994), and (iii) *in situ* hybridization carried out on

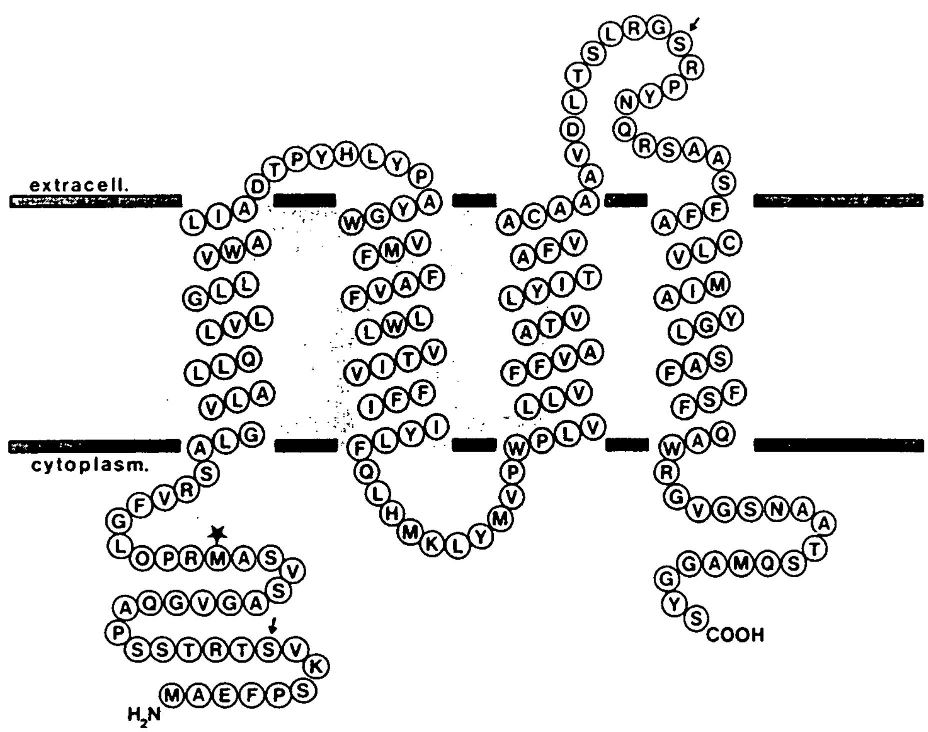

FIG. 8. Proposed topographical model of plasmolipin in the plasma membrane. The putative phosphorylation sites Ser9 and Ser130 are marked with an arrow. A star denotes Met26 as the N-terminus of plasmolipin proposed by Fischer and Sapirstein (1994).

brain sections from P7, 14 and 21 animals and adults showed a developmentally regulated expression that is typical of myelin genes (data not shown). *In situ* hybridization further revealed that other neural cells, like meningeal fibroblasts (Fig. 6A), protoplasmic astrocytes in outer cortex layers (Fig. 6A) and cells of the plexus chorioideus (Fig. 6C), do not express plasmolipin mRNA in detectable amounts.

The analysis of changes in plasmolipin transcript concentration after sciatic nerve injury using two lesion paradigms (crush and transection) is consistent with a role in myelination; (i) in the distal segments of both crushed and transected nerve a marked decline in mRNA was found after lesion, correlating with demyelination in these segments; (ii) in the distal stump of crushed nerve, which is known to regenerate, the transcript level was reinduced, reaching control levels within ~4 weeks after lesion (Fig. 3B); and (iii) in the axon-free distal segment of the transected nerve, where no myelination occurred, the mRNA concentration remained at a low level (Fig. 3B). This time course of plasmolipin expression is in accordance with the previously reported expression of the established myelin genes *MAG*, *P0* and *MBP* (Trapp *et al.*, 1988; Gupta *et al.*, 1990) and *PMP22* (Spreyer *et al.*, 1991).

Further evidence for a role of plasmolipin in myelination was obtained by Northern blot analysis of plasmolipin mRNA steady-state levels during PNS and CNS development. In the peripheral nervous system plasmolipin mRNA levels were undetectable at birth, but increased during further development and reached a maximum at P14–20 (Fig. 3C), thus showing developmental expression very similar to that of the myelin genes *P0* and *MBP* (Lemke and Axel, 1985; Stahl *et al.*, 1990). In the brain, plasmolipin was undetectable at P0, was markedly increased at P13 and reached a maximum at P20 (Fig. 3D), resembling the time course of CNS myelination (Sorg *et al.*, 1987).

Alteration of the intracellular cAMP levels and manipulation of conditions that govern Schwann cell proliferation are widespread methods of studying different stages of Schwann cell development (Jessen and Mirsky, 1991; Jessen *et al.*, 1991). Although the *in vivo* role of cAMP remains controversial, the ability of cAMP to induce myelination in Schwann cell cultures is inversely related to cell proliferation. In dividing cells cAMP induces markers of early differentiation, whereas progression to myelination requires withdrawal from the cell cycle. In our experiments, subconfluent proliferating Schwann cells did not express plasmolipin mRNA and only marginal amounts were detectable after the addition of forskolin. In contrast, the plasmolipin mRNA level was significantly increased in confluent cells that were withdrawn from the cell cycle. This level of expression could be further increased by the addition of forskolin, indicating that plasmolipin behaves like a typical myelin gene.

The biological function of plasmolipin is not known. Tosteson and Sapirstein (1981) reported K^+ conductivity of the plasmolipin proteins after incorporation into lipid bilayers and proposed an *in vivo* function of plasmolipin as an ion channel. It should be noted, however, that the full-length sequence of plasmolipin shows no significant homology to known K^+ channels. Electrophysiological studies of Schwann cell cultures treated with mitogens demonstrated a correlation between the degree of K^+ conductivity and the rate of cell proliferation (Konishi, 1989; Wilson and Chiu, 1993). Given this linkage between K^+ conductivity and cell proliferation, K^+ conductivity appears to be inversely correlated to plasmolipin expression in cultured Schwann cells.

It is remarkable that plasmolipin is not only expressed in the nervous system, where it may play a role during myelination, but also to a high extent in the kidneys. Other glial transmembrane proteins that are involved in myelination are also found to be expressed outside the nervous system, like PMP22 in the lungs and colon (Spreyer *et al.*, 1991), connexin-32 in the liver, pancreas and kidneys (Bennett *et al.*, 1991) and rMAL in the spleen and kidneys (Schaeren-Wiemers *et al.*, 1995), supporting additional functions of these proteins not related to myelination. Whether plasmolipin, like other transmembrane myelin proteins (e.g. PMP22, proteolipid protein and connexin-32), is involved in dysmyelinating diseases of the PNS and/or CNS remains to be investigated.

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Abbreviations

BrdU	5-bromo-2′-deoxyuridine
CNS	central nervous system
DMEM	Dulbecco's modified Eagle medium
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
P	postnatal day
P_0	myelin protein zero
PAGE	polyacrylamide gel electrophoresis
PMP22	peripheral myelin protein of 22 kDa
PNS	peripheral nervous system
rMAL	rat myelin and lymphocyte protein
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
T-MAL	T-cell-specific, maturation-associated protein of lymphocytes

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Plasmolipin: The Other Myelin Proteolipid. A Review of Studies on its Structure, Expression, and Function*

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Proteolipids were discovered by Marjorie B. Lees and Jordi Folch-Pi in 1951 (1) when they isolated the myelin proteolipid protein (PLP). PLP is a major constituent of CNS myelin where it represents at least 40% of the total myelin protein. Since then, proteolipids have been associated with a variety of other membrane domains and organelles (2-4). However with few exceptions (5) all proteolipids described thus far show little homology with each other. In this review we describe the properties of a proteolipid, plasmolipin, which is restricted to specific plasma membranes of kidney and brain (6,7). Plasmolipin is particularly relevant to a Neurochemical Research issue dedicated to Marjorie Lees, since the studies on this protein were inspired by her work on PLP and, like PLP, it is a major myelin protein (8). This review covers our knowledge to date of plasmolipin and describes its structure, localization, development, distribution within the myelin complex, phylogenic profile and putative function.

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* Special issue dedicated to Dr. Marjorie B. Lees.

Abbreviations: CNPase, cyclic nucleotide phosphohydrolase; MAG, myelin associated glycoprotein; MAP-1B, microtubule associated protein 1B; MBP, myelin basic protein; hsp-70, the 70 kD heat shock protein; Na^+, K^+ ATPase, sodium and potassium-stimulated adenosinetriphosphatase; PLP, proteolipid protein.

1) Properties of Plasmolipin in Lipid Bilayers

Plasmolipin was first described in the early 1980s and was originally isolated from canine and bovine kidney plasma membrane (9,10). Originally this protein was referred to as PMPLP, plasma membrane proteolipid protein. Initial purification from canine kidney indicated that this protein exists as two low molecular weight polypeptides now determined to have molecular masses of 17-18 kD. Addition of plasmolipin to lipid bilayers showed that this protein was capable of forming cation-specific ion channels (9). Further analysis indicated that K^+ was needed to be present for channel formation. However, once the channel has formed in KCl it allows the movement of Na^+ as well as K^+ but at approximately 1/3 the rate of K^+. These data distinguish plasmolipin from PLP in that the latter is not cation-selective and channel formation does not require K but requires Na instead (11). These differences indicate that neither protein is forming a channel merely based on their amphipathic characteristics but on a basis specific to the structure of each protein.

The incremental addition of plasmolipin to lipid bilayers indicated two important characteristics (9). First, at low concentrations of the protein the channels were small, showing single channel conductances of about 10pS with no detectable voltage dependency. In con-

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trast, when plasmolipin levels bathing the bilayer exceeded 1 μg/ml, the channels were much larger, 100pS, and exhibited voltage dependency, closing at potentials greater than 30mV. Second, comparison of conductance changes with the molar concentration of plasmolipin added to the bilayer, indicated that these channels were formed by a plasmolipin oligomer comprised of either three or six monomers. Additional studies with purified subunits indicated that both subunits needed to be present for channel formation. Thus, a trimer made of each plasmolipin subunit was a possible channel structure. This ambiguity has in part been answered by structural studies described below.

2) Structural Studies on Plasmolipin

Plasmolipin is typical of proteolipids in that it can be extracted from membranes by neutral chloroform methanol (CM). Once delipidated, i.e., the apoprotein with noncovalently bound lipids are removed, the protein can be converted to a water soluble form by solvent exchange under a stream of N_2. The early structural studies on PLP (12,13) indicated that this conversion led to a marked change in secondary and tertiary structure.

a. Fluorescence Emission. Plasmolipin also exhibits the property of structural flexibility (10). This was initially studied using the intrinsic tryptophan fluorescence which has an emission spectrum between 330-360nm, with the maximum dependent on the polarity of the tryptophan environment. The lower the wavelength (λ) of the emission maximum the more hydrophobic the environment. For plasmolipin, the tryptophan fluorescent emission spectrum in water was a symmetrical peak at 335 nm (Figure 1) indicating that, in water, all the tryptophan residues are buried in an intense hydrophobic field. In CM the protein appears to open up resulting in higher λ emission maxima at 345 and 355nm indicating that tryptophan residues have shifted into more polar environments. Addition of the protein to liposomes gave an emission spectrum similar to that seen in CM indicating that the tryptophan residues of plasmolipin exist both within the bilayer and close to the bilayer surface, exposed to an aqueous (polar) environment. Recent molecular cloning of plasmolipin and deduction of the amino acid sequence predict a distribution of tryptophan residues in the bilayer similar to that inferred from these fluorescent experiments (14).

b. Circular Dichroism. Structural flexibility was also observed in circular dichroism (CD) studies (10). In water the spectrum was indicative of a large hydrophobic core excluding the aqueous solvent and giving a profile dominated by α helix with some β structure and random coil.

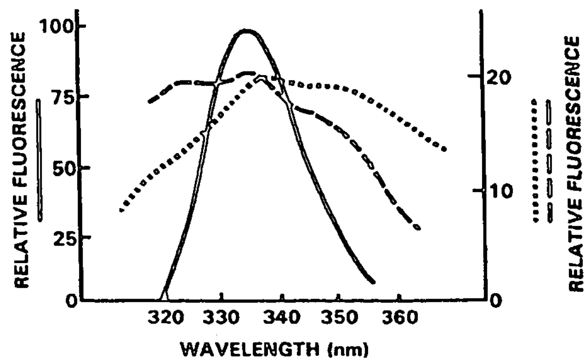

Fig. 1. Fluorescent spectra of plasmolipin in water, Chloroform-Methanol and Liposomes. Excitation in water (—) was at 280nm, with a protein concentration of 0.05 mg/mL. Excitation in CM (---) and in liposomes (▭▭) was at 295nm, with a protein concentration of 0.12mg/ml. The excitation λ does not affect the emission spectra, but was used in CM and liposomes to avoid interference of solvent and lipid. The peak fluorescence in water was arbitrarily taken as 100%. The data in this figure are taken from reference (10) and used with permission of the author.

Table I. Effect of Solvents on Secondary Structure of Plasmolipin

Solvent	% α helix	% β structure	% random coil
H_2O	73	14	12
Phosphatidyl choline/ cholesterol liposome	62	2	35

Insertion of plasmolipin into liposomes gave a significant structural change showing a small decrease in α-helix, a loss of β structure and an increase in random coil (Table I). This is consistent with a structure comprised mostly of transmembrane hydrophobic segments with short extramembranous random coil segments. Based upon its amino acid sequence, the hydropathy plot of plasmolipin shows a similar pattern comprised of four hydrophobic transmembrane segments interspersed with short extramembraneous domains (14) and predicts a general membrane topology as illustrated in Figure 2.

c. Oligomeric Structure. The oligomeric structure of proteins can be inferred by the stoichiometry of binding of specific fluorescent probes such as 8-anilino-1-naphthalenesulfanate (ANS) which has been useful in the study of the quaternary structure of plasmolipin. ANS binds to proteins with 1:1 stoichiometry. Based upon the ratio of [ANS]/[plasmolipin] binding in H_2O it was calculated that plasmolipin exists as an hexamer in this solvent. In CM, it was calculated to be a dimer (10).

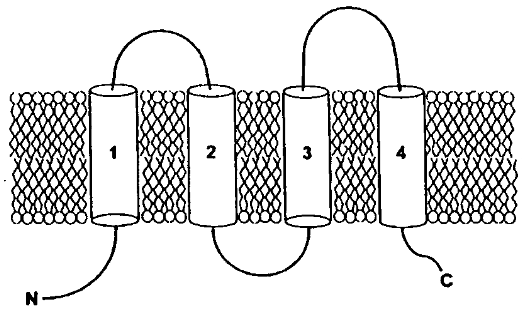

Fig. 2. Membrane topology of plasmolipin. The topological arrangement of plasmolipin within a membrane is derived from the amino acid sequence and hydropathy plot and is depicted as a series of transmembrane segments (hollow tubes) and short extramembranous segments (solid black lines).

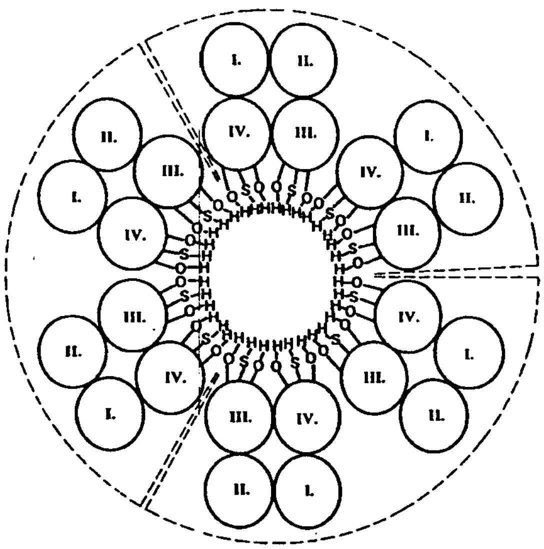

Fig. 3. Proposed quaternary structure of plasmolipin. A quaternary structure of plasmolipin is proposed which consists of three sets of plasmolipin units defined by dashed lines. The plasmolipin unit consists of 2 monomers each containing four transmembrane segments I-IV. The model given is as viewed from the extracellular face. The SH's represent the location of cysteine residues.

These data indicate that two oligomeric states can exist, an hexamer and dimer. The trimeric structure inferred from the in vitro electrophysiological studies, described above, may be an hexamer comprised of 3 sets of dimers. Based on the four transmembrane segments predicted by the plasmolipin sequence we propose a model (Figure 3), that incorporates the concept of a trimer, each made of two plasmolipin monomers, resulting in a protein complex with 24 transmembrane segments, a common motif in K^+ channels (15).

3) Localization and Developmental Expression of Plasmolipin

Plasmolipin is found in brain (16), peripheral nerve (17) and kidney (6), i.e., tissues with a high demand for ion homeostasis to maintain functional capacity. In the kidney, it is restricted to the apical surface of the tubular cells and is concentrated in the proximal tubules (Figure 4A). It differs from Na^+, K^+ ATPase which is present on the basolateral surface (Figure 4B). The levels in kidney increase dramatically during the first four weeks postnatally.

Staining of mixed primary glial cultures (18) with

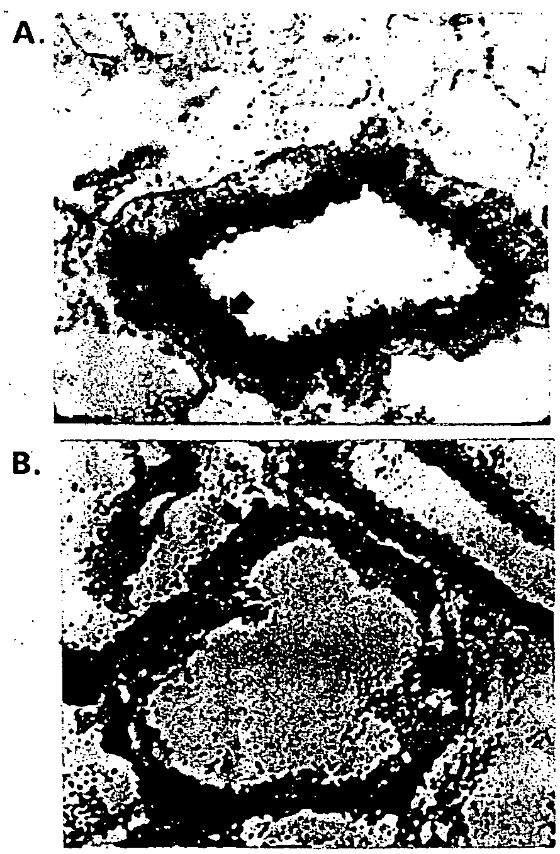

Fig. 4. Immunocytochemical staining of plasmolipin and Na^+, K^+ ATPase in rat kidney. Immunocytochemical staining of plasmolipin (A) and Na^+, K^+ ATPase (B) was carried out as previously described (6). The antibodies to Na^+, K^+ ATPase were used at a 1:500 dilution, and were a generous gift of Dr. George Siegal, Loyola University.

affinity-purified antibodies demonstrated the presence of plasmolipin galactosyl cerebroside (GC) positive cells but not in GFAP positive cells. Plasmolipin staining was first observed at about 8 days in vitro (DIV) as sparse granules of fluorescence in only some of the GC-positive cells. By 14 DIV, plasmolipin staining was intense and evenly distributed throughout the cell body and processes. In mature glial cultures, plasmolipin remained mostly in the cell body and processes, but was not prominent throughout the MBP-positive membrane sheaths (Figure 5). Immunoblot analysis of homogenates from primary glial cultures showed that plasmolipin levels gradually increased between one and four weeks in culture. Similar increases in plasmolipin were found in cultures enriched in oligodendroglia, although the relative levels were higher in the enriched cultures (18). These observations indicate that oligodendroglial cells express plasmolipin during differentiation in culture and predicts its presence in myelin.

Plasmolipin is highly enriched in isolated myelin with quantitation indicating it represents 3-5% of myelin protein in the CNS (7,10). The presence of plasmolipin in myelin has been demonstrated in several ways (8). Immunoblot analysis of different brain regions revealed that plasmolipin levels were higher in regions rich in white matter. Plasmolipin levels in myelin were progressively enriched during five cycles of myelin purification, similar to the enrichment of PLP and MBP. When myelin or brain white matter was extracted with chloroform-methanol the extracts contained, in addition to PLP, a significant amount of plasmolipin. Comparison of CNS and PNS myelin (Figure 6) indicate that plasmolipin is similarly enriched in the PNS (17). Immunocytochemical staining of brain sections showed a restriction of the protein to myelinated tracts with an absence of staining of neuronal cell bodies in the granular and purkinje cell layers (Figure 7). Staining was also absent from the molecular layer indicating that non-myelinated axons do not contain this protein.

Developmental studies in rat brain (6) are also consistent with plasmolipin as a myelin protein with plasmolipin levels increasing more than 7-fold between the first and fourth post-natal week. The biosynthesis of plasmolipin was studied during this period of rapid ac-

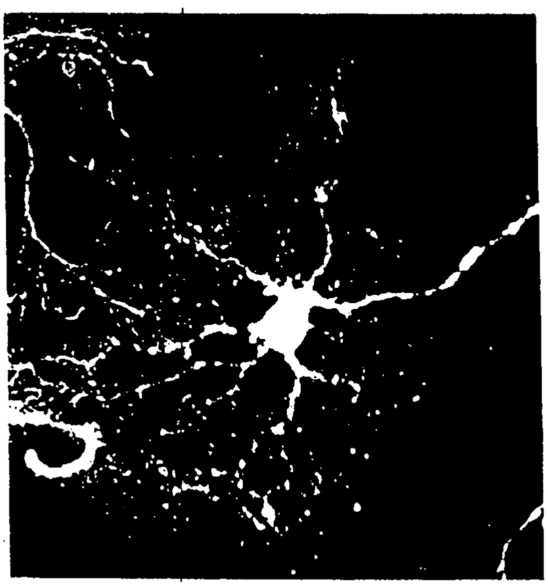

Fig. 5. Immunofluorescent staining of plasmolipin in mouse oligodendrocyte cultures. Enriched oligodendrocyte cultures were prepared and stained by immunofluorescence for plasmolipin as described previously (18) and photographed at 500X.

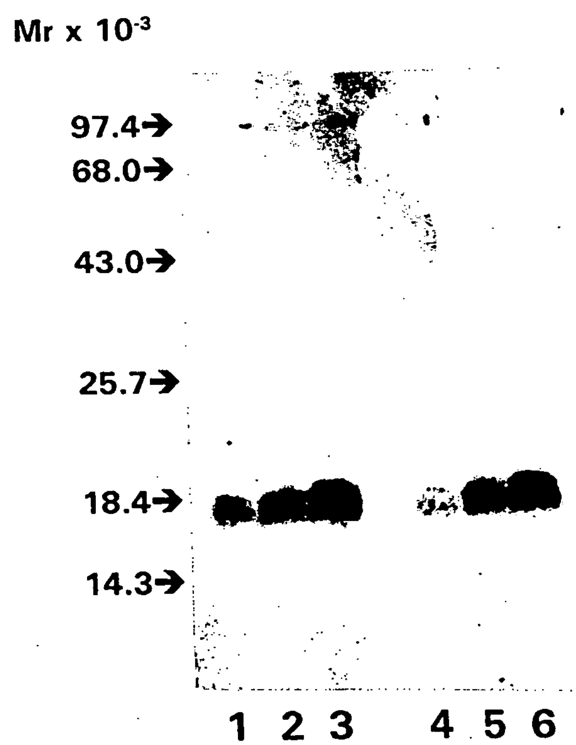

Fig. 6. The level of plasmolipin in purified rat myelin isolated from CNS and PNS. Myelin was isolated and 1, 2 and 5μg protein from CNS (forebrain), lanes 1, 2 and 3, respectively; and PNS (sciatic nerve), lanes 4, 5 and 6 respectively, were analyzed by Western blot. Electrophoresis was carried out on 14% polyacrylamide gels. Under these conditions one should note the separation of plasmolipin subunits. The figure is taken from reference (17) and used with permission of the author.

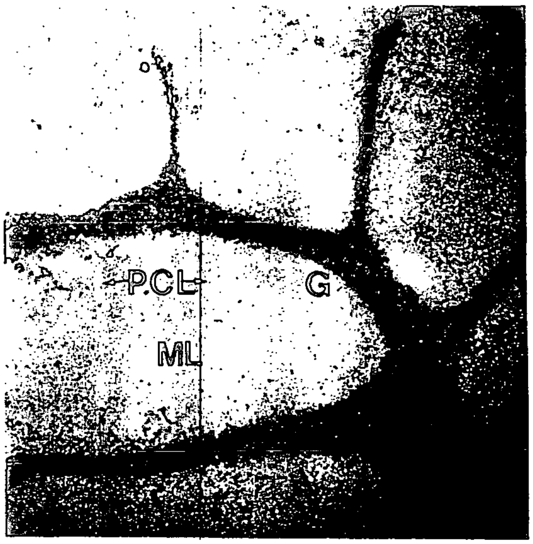

Fig. 7. Immunocytochemical staining of rat cerebellum. The cerebellum was rapidly removed trimmed and frozen in Tissue-Tek OCT media chilled in isopentane and dry ice. The tissue sections (8μ) were placed on gelatin coated slides air dried and stored at −70C. The sections were treated with cold acetone (−20 C) prior to immunocytochemical staining. Endogenous peroxidase activity was eliminated with H_2O_2 and the sections reacted with anti-plasmolipin. The immune complex was visualized with peroxidase-anti-peroxidase as previously described (6). ML is molecular layer; G is the granule cell layer; PCL is the Purkinje cell layer; WM is white matter. Sections were photographed at 100 X.

cumulation. In vitro translation was carried out with free and bound polysomes isolated at postnatal day 16 and plasmolipin purified from the translation mixture by immunoprecipitation. The results demonstrate that plasmolipin is synthesized on membrane-bound polysomes and is inserted into membrane by a co-translational event without evidence of proteolytic processing, i.e. removal of a signal sequence (16).

Subcellular fractionation studies reveal sites of plasmolipin other than compact myelin (7,19,20). Separation of membranes from myelinated nerve tract into fractions highly enriched in compact myelin, periaxolemmal-myelin and axolemma show that plasmolipin is equally distributed among these fractions (7). These data are striking since in the same study proteins such as PLP and MBP were restricted to compact myelin, while MAG and CNPase were highly enriched only in periaxolemmal-myelin. Moveover, axon-specific fodrin, MAP-1B and Na-Channel were all enriched in the axolemmal fraction. These data indicate that plasmolipin exhibits a unique distribution within myelin (compact myelin and periaxolemmal-myelin) and that it appears to become incorporated into the axolemma.

The accumulation of plasmolipin in axolemma may reflect the transfer of this protein from periaxolemmal sites. If plasmolipin were to be transferred to the axon from periaxolemmal-myelin this would not be unique. A mechanism involving vesicle mediated transport has already been postulated for inducible HSP-70 in squid (21) and data suggest a similar mechanism in mammals (22). Similarly, the localization of transferrin to oligodendrocytes in many areas of the CNS (23) suggests that the iron required by the neuron may eneter the neuronal compartment from the oligodendroglial/myelin complex. A vesicular uptake mechanism into the axon has been observed by several investigators who noted endocytotic profiles which enclose periaxolemmal-myelin membrane along with the axolemma (24,25). Purification of clathrin coated vesicles from white matter reveal that plasmolipin is the most abundant intrinsic membrane protein within the vesicle (19). The only other protein present in these vesicles which is unambiguously derived from myelin is the paranodal marker CNPase (19,20). These vesicles are heterogenous with respect to the plasma membrane from which they are derived and probably include oligodendroglial plasma membrane sites as well as periaxolemmal myelin, but electron microscopic evidence indicates the latter is an important source. The enrichment of the axonal cytoskeletal protein MAP-1B (26) on these vesicles indicate further that sites along the axons represent a likely site for the formation of these vesicles. We propose that plasmolipin enters the axon by an endocytotic mechanism and that it becomes inserted in the axolemma when the sorting of the vesicles leads to exocytosis and fusion of the vesicle membrane with the axolemma.

3) Phylogenic Profile

Phylogenic analysis of plasmolipin expression underscores the unique nature of this protein. Using two different polyclonal antibodies plasmolipin was found to be restricted to the mammalian nervous system (17). Western blots of purified myelin protein (Figure 8) showed that mammals but not birds, reptiles, amphibian or fish express an immune reactive plasmolipin. While such analysis has its limitations, i.e., the protein could be present but the dominant epitopes be sufficiently altered so as to preclude reactivity, it should be noted that polyclonal antibodies to the other major myelin proteins show a clear lineage in the CNS going back to amphibians (27,28). If a progenitor to plasmolipin or an homologous protein exists in these lower species, it must

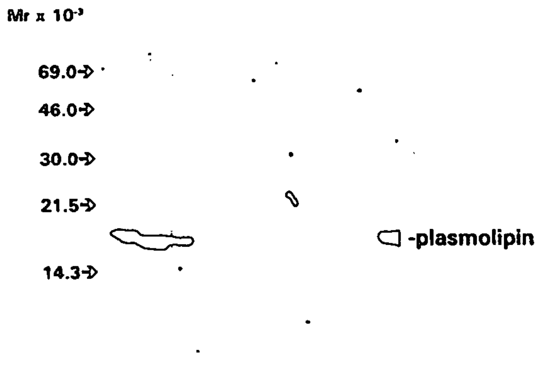

Fig. 8. Phylogenic analysis of plasmolipin in vertebrate myelin. Plasmolipin was analyzed by Western blot. Human, lane A; bovine, lane B; rat, lane C; chicken, lane D; lizard, lane E; turtle, lane F; frog, lane G; goldfish, lane H; shark, lane I; skate, lane J; human PNS; lane K. All lanes contained 10 μg of myelin protein, except the first three lanes that contained 5 μg of rat myelin. Electrophoresis was carried out with 13% polyacrylamide gels. This figure is taken from reference (17) and used with permission of the author.

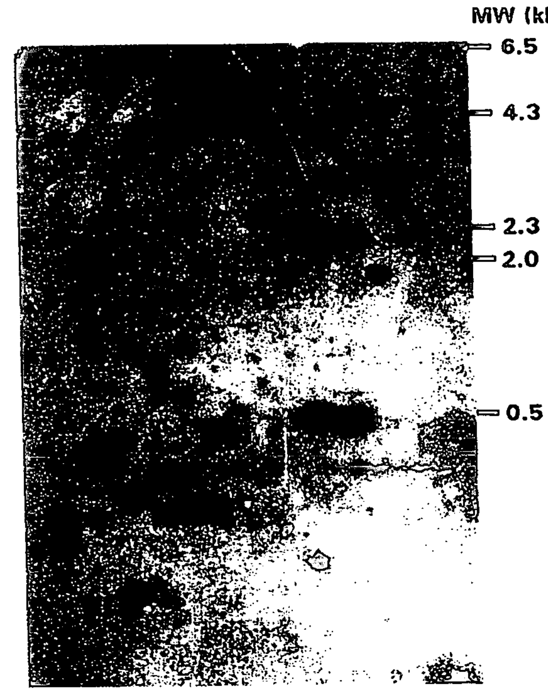

Fig. 9. Southern blot analysis of DNA from various vertebrate species. DNA from different species was digested with Hind III, separated on 1% agarose gels, transferred to nylon membranes and hybridized with a ^{32}P-labeled 1.2kb cDNA probe containing the full coding region of plasmolipin. Lanes 1-6 are DNA from fruitfly, fish, frog, two species of snake and chicken, respectively. Lanes 7-11 are DNA from cow, mouse, rat, chimpanzee and human, respectively.

have been significantly altered so as to abolish antibody reactivity.

The molecular cloning of plasmolipin allowed the phylogenic analysis to be extended to the DNA level. Southern blot analysis with DNA was prepared from mammalian and non-mammalian species and probed at high stringency with a plasmolipin cDNA that included the full coding region. The results showed no detectable homology with non-mammalian species (Figure 9). We conclude that plasmolipin represents a genetic element which is relatively new and not highly conserved. Consistent with this view is the variability in the reactivity of the rat cDNA probe with non-rodent mammalian species reacting strongly with DNA fragments from rats and mice, but weakly and in a different pattern with bovine and primate DNA.

The expression of plasmolipin does not appear to reflect the evolution of the brain per se since an identical phylogenic pattern is observed in the peripheral nervous system (17). Plasmolipin was undetectable in the PNS of species other than mammals where, as illustrated in Figure 7, it is present in levels comparable to that in the CNS suggesting further that its function is unique to mammals.

4) Function

Although we do not know the exact biological function of plasmolipin, its capacity to form voltage dependent K^+ channels suggests that this may be the basis of its in vivo activity. The localization of plasmolipin to renal epithelia and oligodendroglia and its absence from excitable cells such as neurons and skeletal muscle, suggest plasmolipin does not subserve fast K^+ channel activity involved in the control of excitability or neurotransmission. Plasmolipin may therefore belong to a family of slow K^+ channels usually associated with fluid volume and/or intracellular pH regulation (29-31). With the segregation of carbonic anhydrase to myelin, and away from the axon, acidification must be considered as an ongoing process in the myelin complex. The extent of the acidification and its relationship to fluid volume regulation probably depends on a variety of physiological and potentially pathophysiological events. The expression of ion channel proteins which facilitate fluid movement would be important for the maintenance of myelin integrity. In this context, the evolution of plasmolipin and its expression in mammalian myelin may be

of great value. The importance of fluid regulation in the oligodendroglial/myelin complex is underscored by the pivotal role vacuolization plays in most myelinolytic conditions and diseases.

An insight into the control of plasmolipin function in mammalian myelin may come from new molecular evolution data (see Campagnoni et al, this Issue). Dr. Campagnoni data show that the sequence of PLP in the Zebra Finch, differs from the mammalian PLP in 29 amino acid residues. What is striking is that many of these sites reside in the exon where mutations are most deleterious (32). The fact that Zebra Finch have both normal levels of myelin and PLP suggests that PLP mutations in mammals may be deleterious not due to altered PLP structure or function per se but because of other factors which are unique to mammalian myelin. The restriction of plasmolipin to mammalian myelin allows one to speculate that these two proteolipids, PLP and plasmolipin, may functionally interact and that as a result PLP affects plasmolipin function in compact myelin.

Future study on plasmolipin function and the basis for its regulation will clarify our knowledge of this protein and its role in myelin dynamics. If as we postulate, its function is related to fluid volume regulation the study of plasmolipin will be an important focus for understanding the control of myelin integrity.

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Myelin and lymphocyte protein (MAL/MVP17/VIP17) and plasmolipin are members of an extended gene family

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Abstract

An increasing number of four-transmembrane proteins has been found to be associated with CNS and PNS myelin. Some of these proteins play crucial roles in the development and maintenance of the nervous system. In the CNS, proteolipid protein (PLP) is mutated in the myelin disorder Pelizaeus-Merzbacher disease and in spastic paraplegia, while in the PNS, peripheral myelin protein 22 (PMP22) and connexin32 (C × 32) are culprit genes in the most frequent forms of hereditary peripheral neuropathies. Myelin and lymphocyte protein (MAL; also called MVP17 or VIP17) and plasmolipin are additional tetraspan proteins that are highly expressed by myelinating glial cells. However, little is known about the role of these proteins in the nervous system. As a prerequisite for functional genetic approaches in the mouse, we have isolated and characterized a mouse MAL cDNA and the corresponding structural MAL gene. Computer-aided analysis and database searches revealed that MAL belongs to a larger gene family which also includes plasmolipin, BENE and the expressed sequence tag (EST) H09290. While the overall amino acid sequence identities between mouse MAL and the related proteins are relatively low (29–37%), the conserved motif -[Q/Y-G-W-V-M-F/Y-V]- which is found at the junction of the first extracellular loop and the second membrane-associated domain serves as a fingerprint for the MAL protein family. Expression analysis of the members of the MAL gene family indicates widespread expression in various tissues, suggesting a common role of these proteins in cell biology. © 1997 Elsevier Science B.V.

Keywords: Nervous system; Schwann cell; Oligodendrocyte; Tetraspan protein; BENE; Gene structure

1. Introduction

The highly hydrophobic proteolipid protein MAL has first been identified as a marker of human T cell maturation (Alonso and Weissman, 1987) and, based on the extensive alternative splicing of its mRNA, human MAL (hMAL) is thought to exist in multiple isoforms (Rancano et al., 1994a,b). The rat homologue rMAL was isolated by two different screening approaches which were aimed at the identification of either genes specifically expressed by myelinating oligodendrocytes (Schaeren-Wiemers et al., 1995a,b), or proteins which might play a role in intracellular sorting and transport processes during myelination (Kim et al., 1995). Furthermore, the canine homologue cMAL has been described as a component of immuno-isolated transport vesicles from Madin-Darby canine kidney (MDCK) cells (Zacchetti et al., 1995). Based on these results, it was hypothesized that MAL might be involved in vesicular trafficking and cycling between the Golgi complex and the apical plasma membrane.

Northern blot analysis of rMAL mRNA revealed a restricted expression pattern in spleen, kidney, spinal cord, brain and peripheral nerves (sciatic nerve) with the highest mRNA levels found in neural tissues, tightly correlated with CNS myelination (Kim et al., 1995; Schaeren-Wiemers et al., 1995b). Complementary in situ hybridization and immunohistochemical analyses confirmed that in the CNS, rMAL is mainly expressed by

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Abbreviations: aa, amino acid(s); bp, base pair(s); kb, kilobase(s) or 1000 bp; MAL, myelin and lymphocyte protein.

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myelinating oligodendrocytes, but even stronger expression was found in myelinating Schwann cells in the PNS (Schaeren-Wiemers et al., 1995b).

Recently, the myelin-associated proteolipid protein plasmolipin which shares some structural similarities with MAL has been identified and characterized by molecular cloning in the rat (Fischer and Sapirstein, 1994; Gillen et al., 1996). Similar to rMAL, plasmolipin mRNA expression appears to be restricted to kidney and myelinating glial cells of the CNS and PNS. Furthermore, the regulation of plasmolipin mRNA expression during neural development and sciatic nerve regeneration showed a tight correlation with myelination (Gillen et al., 1996). In agreement with these results, plasmolipin has been described as a component of both CNS and PNS myelin. Furthermore, plasmolipin has been localized to the apical surface of tubular epithelial cells in the kidney (Cochary et al., 1990; Fischer et al., 1994). The addition of a crude preparation of purified plasmolipin to lipid bilayers was able to induce the formation of voltage-dependent and potassium-selective ion channels in vitro (Tosteson and Sapirstein, 1981), but the exact functional role of this intriguing protein in neural and non-neural tissues remains to be clarified.

MAL and plasmolipin join a growing group of tetraspan membrane proteins which are associated with myelin. In particular, various mutations affecting the gene encoding the major protein component of CNS myelin, PLP, lead to severe CNS myelin deficiencies in inherited myelin disorders (reviewed by Nave and Boespflug-Tanguy, 1996). In the PNS, the peripheral myelin protein PMP22 gene is mutated in the most common forms of hereditary motor and sensory neuropathies (Charcot-Marie-Tooth disease type 1A (CMT1A); reviewed by Suter and Snipes, 1995a,b), and the gap junction component C×32 has been found to be mutated in X-linked forms of Charcot-Marie-Tooth disease (CMTX; reviewed by Spray and Dermietzel, 1995).

Naturally occurring mutations in animals and humans as well as artificially generated mutations in rodents have improved considerably our knowledge about the crucial role of putative four-transmembrane proteins in neural development, in the maintenance of the normal organism, and in various disease processes (Adlkofer et al., 1995; Suter and Snipes, 1995a; Magyar et al., 1996; Nave and Boespflug-Tanguy, 1996; Sereda et al., 1996). Since the mouse has become the most versatile animal model for the generation of targeted myelin mutants (reviewed by Gu et al., 1994; Kuhn et al., 1995; Soriano, 1995), we have isolated a mouse MAL (mMAL) cDNA and the corresponding genomic mMAL gene as a prerequisite for further studies. The analysis of the obtained sequences revealed that MAL belongs to a distinct subfamily of tetraspan proteins, including plasmolipin, which are widely expressed in various tissues.

2. Material and methods

2.1. Cloning and nucleic acid analysis

Standard methods were used according to Sambrook et al. (1989). A [^{32}P]dATP (Amersham) labeled rMAL cDNA (random-primed DNA labeling kit; Pharmacia) was used to screen a cDNA library (λZAP; Stratagene) from the brain of 8-day-old mice. Positive clones were converted to pBluescript according to the manufacturer's recommendations and sequenced (Sequenase kit, USB) using the following oligonucleotide primers: MALe1f: CGTGTCCAGTCCCAAG; MALe1r: ACTCACAAACGAAGAGC, MALe2f: TCTTTGGAGGCCTGGTG; MALe2r: GTGTGATCCAGGAAGTC; MALe3f: GATGCAGCCTACCACTG; MALe3r: GCGGCGATGTTTTCATG; MALe4r: GCTGACCAGTTAATTGC; MALe4.1: CCAGACTATTCACAAAG; MALe4.2: AGAGACGCTATCCTGGTG; MALe4.3: GTGGGGACAAAGTGAGA; MALe4.4: GCTTGTGTTTTAGTATG.

The isolated mMAL cDNA was used to screen a 129Sv mouse genomic pTCF cosmid library (Pan et al., 1994). Identified clones were analyzed by restriction mapping and Southern blot analysis as well as partial sequencing (using the MAL internal primers and the primers pTCFupper: CCTCAACCTACTACTGG and pTCFlower: AGTGCGGCGACGATAGT). For the determination of the intron lengths using the above exon specific primers, PCR analysis was performed either according to Kogan et al. (1987), or using the long-range GeneAmp XL PCR kit (Perkin-Elmer). Conditions for hot-start PCR reactions (in the presence of AmpliVax beads) were 2 min initial denaturation, followed by 40 cycles of 93°C for 30 s, 55°C for 30 s and 68°C (Kogan protocol) or 72°C (Perkin-Elmer protocol) for 12 min in a thermal cycler (Omnigene-Temperature Cycler; Hybaid, Teddington, UK).

The cDNA clone for HsBENE (Lautner-Rieske et al., 1995; GenBank accession No. U17077) was kindly provided by H.G. Zachau (Ludwig-Maximilians-University, Munich, Germany) and the cDNA for H09290/H09291 (Hillier et al., 1995; GenBank accession Nos. H09290 and H09291) was obtained from the WashU-Merck EST Project. Computer analysis and alignments of sequences were performed using the GCG software package (Wisconsin Open-VMS V.8.0; Madison, WI, USA).

2.2. RNA isolation and Northern blot analysis

RNA was isolated from various organs of adult mice using the guanidinium-isothiocyanate method (TRIzol

kit: Gibco BRL) and analyzed by Northern blotting. The expression of the human cDNAs HsBENE and H09290/H09291 were analyzed on commercially available human tissue Northern blots (Clontech, Palo Alto, CA, USA). An α-actin probe was used to judge the uniformity of the human Northern blots.

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agcgtgtccagtcccaaggccgacgccagctcgcaggc ATG GCT CCG GCA GCG GCT TCG GGT GGC AGC  68
                                        M   A   P   A   A   A   S   G   G   S   10

ACC CTG CCC AGT GGC TTC TCG GTC TTC ACC ACC TTC CCT GAC TTG CTC TTC GTT TGT GAG 128
 T   L   P   S   G   F   S   V   F   T   T   F   P   D   L   L   F   V   C   E   30

TTT GTC TTT GGA GGC CTG GTG TGG ATC CTG ATT GCC TCC TCC CTG GTA CCC TTG CCC CTG 188
 F   V   F   G   G   L   V   W   I   L   I   A   S   S   L   V   P   L   P   L   50

GCC CAG GGC TGG GTG ATG TTT GTG TCT GTG TTC TGC TTT GTG GCC ACC ACT TCC CTG ATG 248
 A   Q   G   W   V   M   F   V   S   V   F   C   F   V   A   T   T   S   L   M   70

ATC TTG TAC ATA ATT GGT ACT CAT GGC GGT GAG ACT TCC TGG ATC ACA CTG GAT GCA GCC 308
 I   L   Y   I   I   G   T   H   G   G   E   T   S   W   I   T   L   D   A   A   90

TAC CAC TGT GTG GCT GCC CTA TTT TAC CTC AGT GCC TCA GTT CTG GAA GCC CTG GCC ACC 368
 Y   H   C   V   A   A   L   F   Y   L   S   A   S   V   L   E   A   L   A   T  110

ATC TCA ATG TTT GAT GGC TTT ACT TAC AAG CAT TAC CAT GAA AAC ATC GCC GCA GTG GTG 428
 I   S   M   F   D   G   F   T   Y   K   H   Y   H   E   N   I   A   A   V   V  130

TTT GCC TAC GTG GTC ACT CTG ATC TAC GTG GTC CAT GCT GTG TTT TCC TTA ATC AGA TGG 488
 F   A   Y   V   V   T   L   I   Y   V   V   H   A   V   F   S   L   I   R   W  150

AAG TCT TCA TAG gacagcagatcgggagctgagacccagatgcaattaactggtcagcccatcttccccattaact 564
 K   S   S                                                                       153

tcctagaacacagactgatggtggagaaaagaaaacaagccaaaaagaaaacaaaacaaacacaaaaacaaaaggaagc 644
catattcaaccatattcgcgtctcttggggtgttatgtttaccttctgtcaaggtttagggcttgctatatttaaccttc 724
tagctaagggaggaaggagttgtcttggaaggggaccttttgcccttgaccgggacagttggtggggaactgggaacct 804
tgatctgagaatgacgatttacacttaccctgaatgtatgtcctaagaatttgcctcttggaatttttaagggctcttt 884
tggagcactcatcccctggatgtcttagtctttgtgaatagtctgggtgtcacaggcactgaaattcacccaacagaggt 964
ctccggacgttcacagtggaagatgagctcctttgaaatgcttcattaaatacttttgtgtctcatgcttcaggtgggga 1044
actggagggatgtctttcattcttgaggaccagggacaccttcctgttcaactttcttcctgcagagtcctttgattaac 1124
ctgattctcaagggacagttcagatgtgtgtgctgggccagtctttcccaaagtgactcactgggacgccaccctcacaa 1204
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tgtctgcagtctcgtgatgtgtttggacattgttctcagaccactccttgtgggagaaagggacctactctacagcaaag 1444
tgagaggtaatggctatctctggactgccacactccaggtgagggatgtctgacctgaaagaagaaagtataattaggaa 1524
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cactagctgatgttttattctccaacaaagtaggagattgagacctacaactaaattagatttggggattttctatgtt 1764
ggttctcaggatggaacccaggggtttgcatgctgggtaagcactcttctaccagatcccatcatcagccccaaattgg 1844
atctcactggcacattcagaaggactttggttatcccagccatactggtatctgaattcaccatttgcccacttaacaca 1924
gccaaaatttcatactaaaacacaagcaaaagattaggggagttattttaaaaattaaaatataaactaagagattat 2004
acaaagtttattattatgtttaaccaaaatctgtaactgagtgctgactggggtagtcttatactttcaacaaagctgaa 2084
ccctaacattacactaaccagcagctcaacacgagtgagatcactttagacaagaagaagaaagagatgaaggaaggaa 2164
gaaaagagggaaaaggaagggtgactaagaggaattgcccaatttctcaatgtttcagattttaatctttaaaagaaac 2244
tttcatacaaaaaaaaaaaaaa                                                            2266
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Fig. 1. Sequence of the cDNA of mMAL. The mMAL transcript contains a 459 bp long open reading frame (indicated by capital letters) which is flanked by a 38 bp long 5′- and a 1775 bp long 3′-noncoding region (sequence submitted to the EMBL nucleic acid database: accession No. Y07626). The aa translation (indicated by the single letter code) predicts a protein of 153 aa with a calculated molecular weight of 16.6 kDa. No consensus sites for potential N-linked glycosylation are present.

A	RnMAL	CfMAL	HsMAL	Hsbene	H09290	RnPlas
MmMAL	91	85	87	36	37	29
RnMAL		87	89	40	38	27
CfMAL			88	38	37	24
HsMAL				41	38	26
Hsbene					42	28
H09290						25

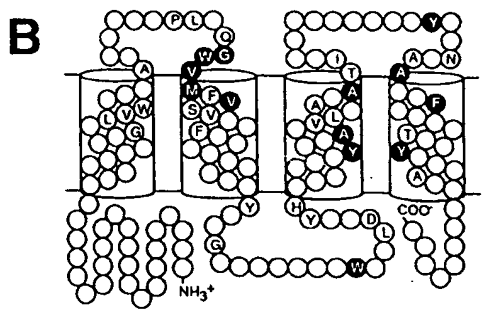


Fig. 2. Quantitative comparison of aa identities and a hypothetical model of the members of the MAL family. (A) Numerical comparison of the members of the MAL family indicates the uncorrected percentage of aa identities as calculated using the Distances program (GCG software package). (B) Schematic representation of the mouse MAL protein indicating the domains conserved within the MAL family. Residues conserved between at least six (out of the seven) members of the MAL family and their known species homologues are lettered. Those residues conserved in all members are indicated by filled circles.

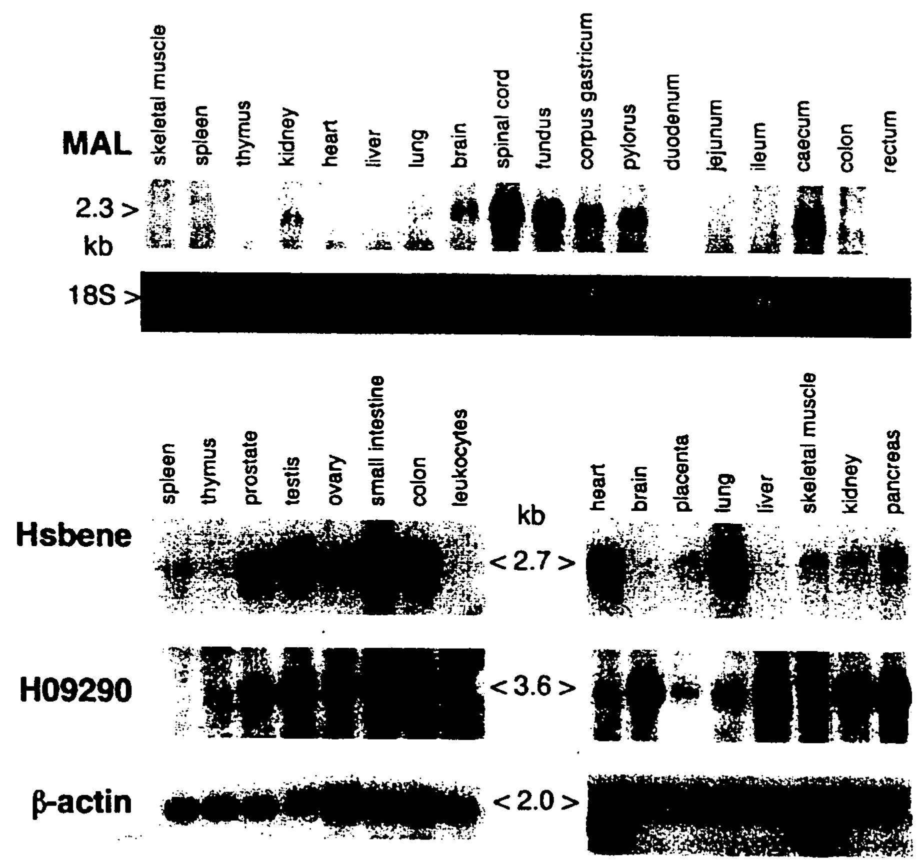
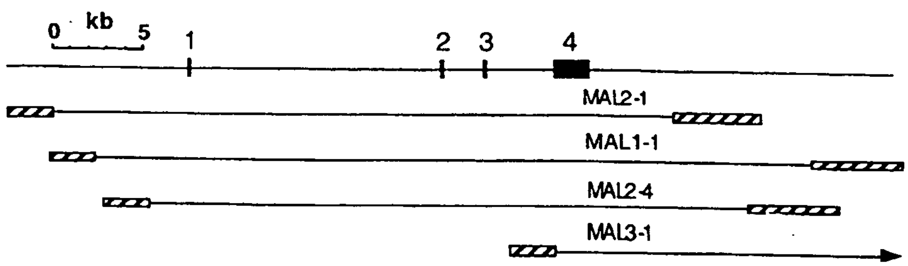


Fig. 3. Northern blot analysis of mMAL, BENE and H09290 expression. mMAL mRNA levels were examined in different tissues of 3-month-old mice. Ethidium bromide staining is shown as loading control. Analogously, the expression of HsBENE (2nd panel) and H09290 (3rd panel) were analyzed using human RNA. Estimated molecular weights of the detected transcripts are indicated. As control, the signal obtained by hybridization of the same membrane with a β-actin probe is shown. A unique 1.8 kb band in heart and skeletal muscle tissues due to cross-hybridization with α-actin is also observed.

A



B

exon number size	exon	intron size	exon
exon 1	...TGT GAG TTT gtgagtgata..	..cctcctgcag	GTC TTT GGA...
>131bp	...cys glu phe	≈14 kb	val phe gly...
	31		32
exon 2	...ATC ACA CTG gtgagtctgg..	..tcacccacag	GAT GCA GCC...
168 bp	...ile thr leu	≈1.7 kb	asp ala ala...
	87		88
exon 3	...GCC GCA GTG gtaagttccc..	..ctggttccag	GTG TTT GCC...
126 bp	...ala ala val	≈3.8 kb	val phe ala...
	129		130
exon4			
>1841 bp			

Fig. 4. Schematic structure of the mMAL gene. (A) The structure of the mMAL gene consists of four exons (filled boxes) and three introns (indicated by a thin line). The isolated and characterized clones with flanking cosmid sequences (indicated as striped boxes) are shown. (B) Exon-intron boundaries of the mMAL gene were determined by direct sequencing of the cosmid clones (sequences were submitted to the EMBL nucleic acid sequence database; accession Nos. Y07627–Y07630). Exon sequences are in capital letters with the aa translation indicated below.

3. Results and discussion

3.1. Cloning and analysis of the mMAL cDNA

The mMAL cDNA was cloned from an 8-day-old mouse brain cDNA library using the rMAL cDNA as a probe. Ten positive clones were isolated by screening approx. 5×10^5 phage plaques. Two clones carried full-length mMAL cDNAs containing the entire coding region of mMAL as well as a short 5′-untranslated region and a relatively long 3′-untranslated stretch (Fig. 1). Comparison of the mMAL cDNA to its known species homologues revealed an overall nucleic acid identity of 86% to rMAL (Schaeren-Wiemers et al., 1995b), 71% to hMAL (Alonso and Weissman, 1987) and 68% to cMAL (Zacchetti et al., 1995). If only the coding regions are included in the comparative analysis, the nucleic acid identities are further increased to 93% (rMAL), 86% (hMAL) and 84% (cMAL). The corresponding interspecies aa identity values range from 91% (rMAL) to 85% (cMAL; Fig. 2A).

Computer-aided databank searches using the mMAL aa sequence as a probe detected several related cDNAs which appear to define a novel gene family. These cDNAs include human HsBENE (Lautner-Rieske et al., 1995), the human expressed sequence tag (EST) H09290 and rat plasmolipin (Fischer and Sapirstein, 1994; data not shown). Hydrophobicity plots suggest that MAL, HsBENE and plasmolipin have similar predicted structures with four potential transmembrane domains, while the EST cDNA clone H09290 is not full length and the putative first transmembrane domain near the amino terminus is missing in the predicted protein (data not shown). Intraspecies comparisons of aa sequences identified HsBENE and H09290 as close relatives to hMAL (aa identities of 41% and 38%, respectively; Fig. 2A). In contrast, the overall aa identity of 27% shared by rat plasmolipin and rMAL is less significant in this type of analysis (Fig. 2A). However, the alignment of a generic MAL consensus sequence obtained from the comparison of MAL species homologues (mouse, rat, human and canine) reveals that plasmolipin is likely to be a distant member of the MAL family (data not shown). In particular, the aa sequence -(Q,Y)GWVM(F,Y)V(S,A)(V,L)- which is located at the presumed junction of the first extracellular loop and the second transmembrane domain appears to be a common denominator (Fig. 2B). Database searches using this aa motif showed that it is exclusively found in members of the MAL protein family (data not shown). The likely structural

and/or functional importance of this motif which is characteristic for this protein family remains to be determined.

3.2. Analysis of mRNA expression

Northern blot analysis was performed to assess the expression patterns of the different members of the MAL family (Fig. 3). In agreement with the results obtained in the rat (Kim et al., 1995; Schaeren-Wiemers et al., 1995b), mMAL is expressed in the spinal cord, brain and kidney. In addition, we found prominent expression of mMAL in the gastrointestinal tract, in particular in the stomach (fundus, pylorus and corpus gastricum) and in part of the large intestine (caecum; Fig. 3).

Similar to mMAL, H09290 is also highly expressed in the brain and the kidney. Furthermore, significant levels of H09290 mRNA are found in the prostate, testis, intestine, heart, placenta, lung, liver and pancreas (Fig. 3). In contrast to mMAL and H09290, HsBENE mRNA is not found in the brain but it is mainly expressed in prostate, testis, gastrointestinal tract, heart and lung (Fig. 3).

3.3. Cloning and analysis of the mMAL gene

The mMAL cDNA was used to screen a mouse genomic cosmid library. Four positive clones were isolated from approx. 9×10^5 colonies (clones 1-1, 2-1, 2-4 and 3-1; Fig. 4A) and subjected to restriction mapping and Southern blot analysis (data not shown). Clones 2-4, 1-1 and 2-1 were found to include 5′-noncoding regions of approx. 2 kb, 5 kb and 8 kb, respectively (Fig. 4A and data not shown).

The sizes of introns 1 (approx. 14 kb), 2 (1.7 kb) and 3 (3.8 kb) were determined by long-range PCR, and the splice acceptor and donor sites of all introns were identified by direct DNA sequencing. All exon-intron boundaries were shown to be in agreement with the -GT...(5′ donor)...AG- (3′ acceptor site) rule (Fig. 4B).

4. Conclusions

(1) MAL, plasmolipin, BENE and the EST clone H09290 define a novel gene family which is widely expressed in various mammalian tissues suggesting a basic role of these related molecules in cell biology.
(2) The members of the MAL/plasmolipin/BENE H09290 family share 29–37% aa identities and have similar predicted structures as tetraspan membrane proteins.
(3) The shared aa sequence -(Q,Y)GWVM(F,Y)V(S,A)(V,L)- which is uniquely found in this protein family may serve as a molecular fingerprint for the identification of members of this family.

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